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SYMPOSIUM

ON

GENETIC APPROACHES TO

SOMATIC CELL VARIATION

PUBLISHED BY

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

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1958

SYMPOSIUM

ON

GENETIC APPROACHES TO SOMATIC CELL VARIATION

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DEDICATION

At the closing meeting of the Research Conference at Gatlinburg on April 5, the following resolution was passed:

> "The participants and guests attending the Eleventh Biology Research Conference held at Gatlinburg, Tennessee, send our best regards to Professor Richard Goldschmidt on the occasion of his eightieth birthday. We honor him in his leadership, honesty, and strict attendance to individual approaches along the lines that he, himself, developed and that have now proved of such great importance. Our respects to Professor Goldschmidt!"

This resolution reached Professor Goldschmidt just a few days before his death.

We dedicate this volume to Professor Goldschmidt, whose leadership in genetics has influenced this field along lines that have now proved to be so important.

ALEXANDER HOLLAENDER

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INTRODUCTION

The Eleventh Annual Research Conference, "Genetic Approaches to Somatic Cell Variation," was held in Gatlinburg, Tennessee, April 2–5, 1958. This conference grew out of the previous one, "Antibodies: Their Production and Mechanism of Action." It was based on the premise that the implications of an immunological approach to modern biology are particularly important to genetics. The usefulness of this approach was emphasized and its significance to somatic cell variation in radiation work and studies of other uncontrolled developments of the cell were stressed. The discussion included the points of view of geneticists, immunologists, biochemists, and investigators in modern cancer research. Although no conclusions were reached, the importance of further research in these difficult fields becomes obvious from reading this report.

The conference, as the previous ones, was sponsored by the Biology Division of the Oak Ridge National Laboratory and the Division of Biology and Medicine of the Atomic Energy Commission. As in previous conferences, the free and open discussion was especially valuable in pointing up interdisciplinary implications of this field. Much of the discussion is reproduced in this volume.

A committee composed of D. L. Lindsley, K. C. Atwood, and A. D. Conger was responsible for arranging the program of this conference. Dr. Lindsley also assisted in the over-all organization of the meeting. We are indebted to Drs. D. L. Lindsley, Ray D. Owen, Drew Schwartz, and G. David Novelli for valuable advice in the preparation of this volume.

Previous symposia in this series are:

1948 — Radiation Genetics

1949 — Radiation Microbiology and Biochemistry

1950 — Biochemistry of Nucleic Acids

1951 — Physiological Effects of Radiation at the Cellular Level

1952 — Some Aspects of Microbial Metabolism

1953 — Effects of Radiation and Other Deleterious Agents on Embryonic Development

1954 — Genetic Recombination

1955 — Structure of Enzymes and Proteins

1956 — Biocolloids

1957 — Antibodies: Their Production and Mechanism of Action

ALEXANDER HOLLAENDER

ERRATA

Page 142, table 6. The third and fourth lines should read —

MNSB	High	None (40)	—	—	None (33)	—	—
MSWC	Very low	71 (76)	Not specific	29 (35)	78 (78)	Not specific	70 (135)

Page 216. The word "serotinin," in 27th line should read serotonin.

Page 241. Table 1. The second column, fifth line should read —

FAK I

Page 244. Table 4. In line 3, for primary 2a, the number of cells under 41 should read 4.

Page 333. Formula should read —

$$\rightarrow C \rightleftharpoons D \rightleftharpoons E \rightleftharpoons F \rightleftharpoons G \rightleftharpoons H \rightleftharpoons$$

THE NUCLEUS AND SOMATIC CELL VARIATION [1]

CURT STERN
Department of Zoology, University of California

INTRODUCTION

Somatic cell variation in all multicellular organisms is of the essence of development. Differentiation implies somatic cell variation. Its emergence has been the subject of discussion and experiment for at least three quarters of a century. Are the variations of cells that do occur irreversible, or are they reversible and only superimposed on basic unchanged properties? Are the variations anchored in the cytoplasm or the nucleus of the cell? Somewhat arbitrarily, but after all in conformity with the actual morphological and physiological partition of cells into nucleus and cytoplasm, the first two reports of this symposium have divided the general topic of somatic cell variation according to the nucleus-cytoplasm division line. My survey deals with nuclear aspects.

The fundamental dilemma of developmental genetics is well known. For a short period, before mitosis and meiosis had been fully appreciated and before the rediscovery of Mendel's work, it seemed possible to assign intrinsic differences to the nuclei of different tissues. The concept of a system of regular succession of genetically unequal divisions, which like prearranged clockwork separate the manifold potencies in development and thus assign each type of tissue its proper endowment, provided a formal framework for the understanding of the role of the nucleus in cell variation during development. The concept was a fruitful one. It led to experimental

[1] Supported by a grant of the National Science Foundation. I acknowledge gratefully the help given by Dr. Aloha Hannah-Alava in the preparation and review of this manuscript.

tests. They did not seem to support it. The result was the apparent paradox of the obvious fundamental differences among differentiated cells, in spite of an apparent invariance of the nuclei with their fundamental endowment of the genetic material. The paradox was not insuperable. If all genes are present in all nuclei we could assume that only some genes, different ones from cell type to cell type, were active at any stage. Differential activation of genes ordered in space and time provided a framework within which theory and experiment could advance.

Somatic cell variation is not limited to normal developmental differentiation. Variations within a given tissue of cells arise that may be compatible with its established type of differentiation or may introduce fundamentally new properties. Thus the nuclei of the cells of mammalian livers may assume different sizes, owing to their acquiring different numbers of chromosome sets, but the cells remain liver cells. On the other hand, the nuclei in cells of many tissues may change, and correlated changes of cell growth will lead to new and not infrequently tumorous properties.

Somatic cell variation is not restricted to differentiated multicellular forms. Vegetatively growing fungi may consist of uniform cells. Somatic variations in type of growth, color, biochemical function, and sexual potencies, the latter observed when sexual differentiation sets in, have long been known and have been analyzed more recently in *Neurospora, Aspergillus,* and other genera. Bacterial variation, outside of sexually accomplished changes, is of equally long recognition.

Still another type of somatic cell variation is known or suspected from those multicellular organisms that reproduce asexually by budding from meristematic tissues, such as runners of plants, or in fission of flatworms, annelids, and other animals. The best evidence of variation comes from artificial propagation of plants. Bateson ('26) has given numerous examples of different species in which some cuttings from roots grow into plants different from the parental plant in flower color, development of the reproductive organs, leaf

shape, and other traits. Bud variations (sports) so frequent in plants belong in the same group of phenomena. Even the great variety of forms in such species as the dandelion (*Taraxacum officinale*) in which reproduction occurs by apogamy, i.e., development from a somatic cell, may well be traced to somatic cell variation.

The primary reason that the topic of somatic cell variation has lately come to the foreground is its relevance to the study of tissue cultures *in vivo* and *in vitro*. Particularly, successes in raising clones of mammalian, including human, cells have led to new observations of somatic cell variation under controlled conditions. They have raised hopes of developing an approach to the genetics of man that could hardly have been foreseen even a few years ago, and that seems to encompass great possibilities of both fundamental and applied nature.

TYPES OF NUCLEAR VARIATION

Germ line versus soma

Before restricting ourselves to consideration of nuclear variation in somatic cells, it is proper to refer to the discoveries of cytologically clear-cut differences between the nuclei of somatic cells and those of germ cells of certain animals and plants. The significance of the case of the *Ascaris* chromosomes has been variously interpreted. Here the nuclei of the germ line receive replications of all chromosomal material that entered the fertilized egg, whereas the somatic nuclei, in consequence of an elimination process known as "diminution," lack the end sections of the original chromosomes. When first discovered by Boveri (1887), the *Ascaris* situation seemed a paradigm of a general nuclear differentiation between germ line and soma. Then, when hardly another organism with such visible differentiation seemed to exist, the interest in *Ascaris* declined. More recently a good many species have been shown to have different chromosome constitutions in germ cells and soma (see Reitberger, '40; White,

'50; Beermann, '56). In the dipteran families of the cecidomyidae, sciaridae, and chironomidae, early in embryology, and in different ways, the future somatic cells eliminate as many as 66 chromosomes, or in *Cardiocladius* even about 80. In *Cardiocladius* the contrast between the nuclei of the future germ line and those of the soma is extreme — about 82 in the former, only two in the latter. This, however, is not all. Later development of the germ line involves partial duplications and eliminations of the about 80 "supernumerary" chromosomes. As a result, cells originate that have no supernumeraries and others that have the full number. Cells without them become nurse cells in the female and degenerating spermatocytes in the male. Those with supernumeraries develop into the functional gametes.

It must be admitted that such visible chromosomal differences between soma and germ line, though more frequent than realized earlier, are yet restricted to a small minority of living forms. Nevertheless, their significance poses a general problem. Moreover, supernumerary chromosomes are met with sporadically in a variety of organisms other than insects. The B chromosomes of maize and rye are examples and so are those of the grass *Poa alpina* (Müntzing, '48). The latter example is important since the extra chromosomes are absent in the root cells of plants whose germinal cells contain from two to eight such chromosomes.

It should be pointed out that the terminology, which applies the word "supernumerary" alike to all these special chromosomes, needs improvement. In the insect groups cited, supernumerary chromosomes are named so because they are absent in the somatic tissues and obviously are not needed for somatic development. They are nevertheless essential components of the germ line. In maize, rye, and *Poa*, as well as in some animals, the supernumerary chromosomes occur only in some individuals and often in different numbers per individual. Such true supernumeraries are not only nonessential for development of germ cells but also may actually be disadvantageous. In rye, for example, too great a number

of supernumeraries leads to abnormal spindle formation and ensuing sterility (Håkansson, '57). It may be useful in the future to distinguish between the nonessential supernumerary and the necessary and germ-line limited chromosomes (see Metz, '38). The adjective "accessory," which has also been used for supernumerary chromosomes, should be avoided since it was historically applied to the X chromosome.

In some organisms, cells are differentiated from one another by presence or absence of proved or probable Feulgen-positive material that forms part of the chromosomes or presumably is derived from them. The material of the ends of the undiminuted *Ascaris* chromosomes furnishes a clear example. Another is the curious ring of "chromatin" that, in the metaphases of the oogonial divisions in *Dytiscus* and related water beetles, passes into the future oocyte cells but not into the nurse cells. Similar phenomena in crane flies have recently been extensively studied by Bayreuther ('56).

It is a general characteristic in all these situations that the germ cells possess chromosomes or chromosome derivatives that are absent in other cells. There are, however, observations that show that the nuclei of mature germ cells may contain less "chromatin" than earlier stages. In the moth *Solenobia*, Seiler ('14, '23) described how, during the first and sometimes also the second meiotic division of the egg, each chromosome pair secretes or sheds a large stainable droplet that later becomes resorbed in the cytoplasm. The eliminated material in this case contains ribonucleic acid (Ris and Kleinfeld, '52). Several other lepidoptera and a coelenterate (Weill, '24) show a similar "chromatin" elimination.

Nothing comparable to the visible chromosomal differentiation between germ line and soma or within the germ line between future gametes and nongametic cells has been found between different somatic tissues. If such differentiations should become known, they should not be accepted automatically as causal agents in somatic cell variation. It has never been established that differentiation of the germ cells of *Ascaris* is dependent on the undiminuted state of their nuclei.

It is quite possible that the specific cytoplasm of these cells — which Boveri showed to be the decisive factor in keeping the chromosomes intact — is the essential determiner of germinal differentiation, or that both the nuclear integrity and the specific cytoplasm are necessary.

In certain males of the fly, *Trichocladius,* Beermann ('56) demonstrated that the presence of supernumerary chromosomes is not responsible for normal spermatogenesis as opposed to degeneration of those cells that have no supernumeraries. He reports on individual males that lacked supernumerary chromosomes altogether: "Yet, the premeiotic gonial division is clearly differential, not with respect to chromosomes this time, but still with respect to the potencies of the daughter cells. Only one of them carries out normal spermatogenesis; the other regularly degenerates . . ." Clearly, the differential cell division that results in two cells with very different fates is not based on any visible nuclear differentiation.

Nuclear variation of genetic nature in somatic cells

Somatic crossing over. In special cases mitosis, in a cell heterozygous for a given locus, *Aa,* can lead to the formation of two homozygous daughter cells, one *AA* and the other *aa.* This is brought about by somatic crossing over between two of the four chromatids of the relevant homologous chromosomes. Their kinetochores behave in typical mitotic fashion. Each kinetochore divides; the sister kinetochores, each carrying a chromatid, move to opposite poles. The two nonsister kinetochores may, as a result of crossing over, carry sections derived from sister chromatids to the same pole; hence *AA* may become incorporated in one nucleus and *aa* in the other. This was the explanation derived for the presence of phenotypically *aa* spots on the surface of *Aa* individuals of *Drosophila* (Stern, '36). It was based especially on the analysis of twin spots in which, on *Ab/aB* individuals, the recessive phenotype *aa* appears in an area adjacent to one with the recessive

phenotype *bb*. Evidently, somatic crossing over between the *B* locus and the kinetochore, followed by mitosis, had led to sister cells with the genotypes *aB/aB* and *Ab/Ab*. Further multiplication of these genetically variant cells had led to formation of two adjacent variant areas each consisting of many cells.

Somatic crossing over could easily have been regarded as a peculiarity of Diptera, in which close pairing of homologous chromosomes is the rule in both meiotic and somatic cells. But results akin to those from somatic crossing over in *Drosophila* have been discovered by J. Lederberg ('51) in *Escherichia coli,* with its presumed cytologically ill-defined chromosome equivalents, and occurs so abundantly between the chromosomes of diploid somatic nuclei of *Aspergillus nidulans* and related fungi that it has been made the basis of mitotic mapping of genic loci (Pontecorvo and Käfer, '56, '58; Käfer, '58). It is likewise of frequent occurrence in diploid yeast cells (James and Lee-Whiting, '55; Roman, '56). Cytologically, some degree of somatic pairing has been described in various organisms. It seems to be of regular occurrence in the butterflies and moths.

With our ignorance of the basic events in crossing over, we may hope for somatic crossing over in cells of human tissue cultures. It would provide a tool for the discovery and analysis of genetic linkages in man. Perhaps much more important, because of homozygosis resulting from somatic crossing over, recessive genes carried in heterozygous combination could first reveal their presence and thus become available for study — regardless of whether the recessives were present in the individual who served as cell donor or whether they arose, by mutation, later in the history of the culture.

Mutation. Mutations in a multicellular organism may occur in any of its cells and at any stage in the life cycle. If the mutant genotype expresses itself in descendants of a mutated somatic cell, then a mosaic individual appears that exhibits the original phenotype in some parts and the new phenotype in others. Usually, the interpretation of such a mosaic as at-

tributable to mutation rests solely on phenotypic similarity with known mutants. It is not subject to direct test by evidence for Mendelian transmission since, by definition, somatic cells do not participate in sexual reproduction. If, however, mutation in an animal takes place early enough in development, before the germ line and soma separate, then not only the soma but also the gonads may consist of a mosaic of mutant and nonmutant cells. Such situations have been found in *Drosophila* and laboratory mammals and conclusive proofs, by breeding tests for occurrence of early embryonic mutations, have been furnished. In plants, where the separation of reproductive from many somatic tissues may occur throughout life at many regions of growth and differentiation, the mutational nature of bud variations has often been demonstrated. In those microorganisms in which sexual processes are known, proof that somatic mutations do occur can easily be established. In haploid yeast, the appearance of "petite" colonies whose restricted growth results from an inherited respiratory deficiency is based sometimes on extranuclear, sometimes on nuclear, variation (Ephrussi, '51). The distinction between nuclear and extranuclear inheritance is possible because yeast can be crossed. Unlike the extranuclear change, the nuclear change segregates in Mendelian fashion and thus proves that the somatic variation involved a nuclear chromosomal element. The same conclusion has been reached for variations occurring in vegetative cultures of *E. coli.*

Mutation in diploid cells affects only one member of a pair of alleles. If the mutant is a recessive and the cell a somatic one, phenotypic expression of the mutant must wait for mutation of the other allele, for somatic crossing over (which leads to homozygosis), or for other processes with similar consequences. These will be discussed. It is doubtful, however, that strictly recessive genes exist at all. In many cases, by detailed morphological, physiological, and particularly biochemical studies, we are able to distinguish between the "dominant" type of homozygous individual and that of

the closely similar but not identical heterozygote [e.g., leaf shape in the nettle *Urtica* (Correns, '18); numerous traits in man (Neel, '53)]. It may therefore be expected that, even in cultures of diploid cells and in cultures with perfect chromosome behavior, occurrence of recessive mutations may be demonstrable.

Point mutation and chromosome aberration. The nature of genetic changes has played an important role in the history of genetics. The word "mutation" was first used in experimental studies by de Vries ('01). Much later it was shown that some of his newly arisen mutant Oenotheras were the results of a variety of gross chromosomal variations, namely, different degrees of polyploidy and heteroploidy. Others were segregation products of the complex heterozygotes that many Oenotheras proved to be.

The problem of whether a genetic change, aside from segregation and recombination, belongs to the category of chromosome aberration in which whole chromosome sets, whole chromosomes, or appreciable parts of them have been added to or subtracted from the normal complement or whether it belongs to the intragenic change called "point mutation," has never left the geneticist. Recognition of a chromosome aberration rests on the positive evidence of microscopic identification, but a point mutation is defined only by the negative aspect of its being a change not microscopically visible. This seemed, at first, merely a matter of the limits of optical resolution, but uncertainty about discreteness or continuity of genic material in chromosomes and the existence of position effects on genes have led to conceptual as well as observational difficulties.

Nuclear changes involved in somatic cell variation also range from visible to invisible without a distinct borderline between them. Nothing essentially new can be said about the fuzzy area of invisible changes, but a great amount is known about chromosome aberrations.

Ploidy. The schematic picture of a whole zygotically diploid organism consisting of chromosomally identical diploid

somatic cells requires modification. Tetraploid, octaploid, or other polyploid cells occur regularly in otherwise typically diploid tissue, even in those as different as mammalian liver and angiosperm roots (Geitler, '53). Differentiation of certain secretory cells, including nurse cells in insects and other invertebrates, hair cells in higher plants, bristle organs in insects, and scales on moth wings, is invariably correlated with polyploidization of the nuclei, occasionally of extremely high order. Sometimes, perhaps the majority of the somatic cells of the organism are polyploid, as in the larvae of Diptera in which most of the mitotically active cells that persist into or build up the adult state (e.g., those of the ganglia and imaginal discs) remain diploid while most of the nondividing but growing cells of the larval body multiply their chromosomes endomitotically. The same processes are found in zygotically haploid individuals. The parthenogenetically produced initially haploid male larvae of the honeybee increase the nuclear content of most somatic cells to diploidy and polyploidy (Risler, '54; Merriam and Ris, '54). In general, the reduplicated chromosomes of polyploid nuclei remain as separate bodies, sometimes located near one another, sometimes dispersed throughout their volume. In Diptera with somatic pairing of homologous strands, cable-like polytene chromosomes are built up, most familiar are those from the salivary gland chromosomes.

During a mitotic cycle, replication of chromosomes uniformly encompasses all chromosomes of a nucleus. The same is true for endomitoses although rare exceptions have been postulated [e.g., Schultz ('41) reported a lower rate of multiplication of the Y chromosome than of other chromosomes in the nurse cells of *Drosophila melanogaster*]. If, in general, each endomitotic step doubles the number of all chromosomes, it becomes a problem to account for triploid, pentaploid, and other uneven states of ploidy as well as for even states such as hexaploidy or decaploidy that are not part of the series 2^x, where x is a whole number.

It has been customary to invoke the action of multipolar mitoses for the accomplishment of a lowering of chromosome numbers from tetraploid to triploid and from other higher to lower states. Multipolar mitoses distribute chromosomes at random (apart from segregation of sister chromosomes). They are highly inefficient in accomplishing segregations of whole genomes. The role of multipolar mitoses in leading to the production of triploids and other ploidy states different from 2^x is thus not obvious particularly in species (for example, mammals) with a large number of chromosomes per haploid set.

The apparent dilemma would disappear if so-called triploid and other polyploid chromosome numbers different from 2^x were, in reality, not polyploids with full multiples of the haploid set. Heteroploid cells, in which the presence of some specific chromosomes in excess of a trisomic condition and of others below it add up to three times the haploid number, would not be triploids. Such heteroploid constitutions rather frequently could be the result of multipolar spindles. It may well be that many "forbidden" ploidy states can thus be explained away, but it is doubtful that this can be done generally. If all cells with apparently polyploid chromosome numbers other than 2^x were actually heteroploid, a great many cells with a variety of chromosome numbers other than multiples of the haploid number n should also be present. This does not seem to be the case in the cells of rat livers, according to Gläss ('56, '57). Although not fully explicit in this respect, this investigator, who reports frequent ploidy, from $1n$ to $6n$, and who encounters some cells with from $7n$ to $10n$, implies absence or rarity of heteroploid chromosome numbers (a single slightly hypodiploid cell is referred to and studied in detail). Gläss also states that the frequency of multipolar mitoses in his material is much too low to account for the high frequencies of cells with forbidden multiples of haploid sets. He calls for other means of production of such cells and believes he has discovered a phenomenon essential for their action. This is "Genomsonderung," genome segrega-

tion. Among more than a thousand metaphase stages of diploid cells, 9–14% had the two haploid sets of 21 chromosomes grouped separately from each other. In triploid metaphases, 27–41% had separate genome groupings of the types $2n$-$1n$ and $1n$-$1n$-$1n$, and similar high frequencies of nonrandom separations were counted in tetraploid and higher polyploid cells. Gläss analyzed the karyotype of his rats and characterized, to some extent, the different chromosomes by length, position of kinetochore, and differentially staining segments. His designation of chromosome groups as genomic (e.g., haploid, diploid) is not based simply on number but also on individual recognition of chromosomes. Unfortunately, the papers quoted do not provide data on the total number of cells in which such detailed determination was possible. It must also be added that the cells were studied in squash preparations. One wonders in looking at the microphotographs how much the pressure may have artificially separated presumed genomes. One wonders too whether some of the imaginary lines drawn to indicate the borders between genomes could not have been drawn differently, either to include some of the chromosomes in a group or to exclude them.

But such doubts cannot be applied to similar observations in a very different group of organism, the weevils (curculionidae). Many species in this family of beetles are polyploid and parthenogenetic. When the unpaired chromosomes arrange themselves for the first (and only) nuclear division of the maturing egg, they may appear on a single plate as in somatic mitoses, irregularly arranged as if for a multipolar spindle, or on different metaphase plates in groups that contain exact multiples of $1n = 11$ (Suomalainen, '40, '47). In triploid species, separations into groups with diploid and haploid numbers were seen, and, in a tetraploid species, distributions of the three types $3n$-$1n$, $2n$-$2n$, and $2n$-$1n$-$1n$ occurred in 18 of 40 eggs. These observations are based on sectioned material, and the separation of groups of chromosomes on separate metaphase plates undoubtedly is not an artifact.

If genome separation in somatic cells is a real phenomenon — further evidence is highly desirable — then it may be associated with the origin of forbidden types of polyploid nuclei. Gläss cites various authors who assume the occurrence of constrictions in the interphase nuclei of liver cells leading to amitotic separation of nuclear parts. If the chromosomes of whole genomic sets have a tendency to lie in separate groups, amitoses might separate them. Thus, from a tetraploid nucleus, could come one haploid and one triploid nucleus, both constitutions not belonging to the series 2^x.

Gläss also considers the possibility that separated genomes or groups of genomes in the same nucleus may behave differently in respect to endomitosis, one group, for instance, reduplicating itself while another remains unchanged (see also Viveiros, '51). Schemes of such differential multiplication could be devised that would account for all types of ploidy in somatic cells. Evidence for such asynchrony in the duplication of chromosomes is hardly available. In lepidopteran cells, slight differences in timing of endomitotic division of chromosomes located in different parts of polyploid nuclei have been seen, but no skipping of a whole cycle by any chromosome has been noted (Lipp, '55).

The idea of genome separation is rather foreign to cytologists and geneticists. Independence of chromosomes from one another within a set is not only a frequent observation but has practically become a postulate. A few phenomena have, however, long been bothersome to those who subscribe to this postulate. Sometimes, separate chromosomes apparently tend to form collective entities, "Sammelchromosomen," and classifying these structures as artifacts has not always been convincing. Then, there is the mysterious property of the three autosomes that the *Sciara coprophila* male brings into the egg of his offspring. All through the development of this fly, these chromosomes reside in the same nuclei together with and indistinguishable from their homologs that came from the female parent. Together they divide mitotically. When meiosis begins in the testes of the male offspring, homologous chro-

mosomes pair and become arranged on the first meiotic spindle. The division of the spermatocyte is unequal. One set of chromosomes remains in the main cell, which will continue to differentiate into sperm; the other set accumulates in a small cellular bud, which degenerates. Cytologically, the two segregating chromosome sets are alike in appearance. The postulate of independent recombination would demand that each be a mixture of maternal and paternal chromosomes. But genetics teaches otherwise. Crosses with genetic markers show that no paternal and all maternal genes are transmitted. Unless one wishes to make other unproved assumptions — for instance, complete and unidirectional Winklerian conversion — one must conclude with Metz ('38) that the two parental genomes retain, for a generation, specific properties "imprinted" on them by their having in the preceding generation been located in a male or a female. These properties make the genomes segregate to opposite poles, specific for the paternal and maternal sets.

A similar though not quite so striking instance of retention of common properties of a whole set of chromosomes has been described in the scale insect *Pseudaulacaspis pentagona* (Brown and Bennett, '57). In this species the fertilized egg receives eight chromosomes from each parent. In female embryos all 16 chromosomes are regularly transmitted by mitoses to the somatic cells. In male embryos eight chromosomes are eliminated from all cleavage nuclei (except for a special polyploid sector). The eliminated chromosomes are of paternal origin only as proved by marking experiments in which X irradiation of either parent produced chromosome fragmentation. Fragments were eliminated only when they came from the father.

There are a few examples where single chromosomes or chromosome parts behave differently according to whether they came from the male or female parent. In *Drosophila melanogaster,* a ring X chromosome is preferentially eliminated from the egg nucleus if introduced through the sperm instead of coming from the egg (A. Hannah-Alava, unpublished). And according to Prokofyeva-Belgovskaya's account

('47), the frequency of heterochromatization of a certain chromosome region again depends on its parental source.

The relative segregation of all chromosomes of a set in rat liver cells is not the ultimate in "belongingness." Gläss finds cells that, within the haploid set of 21 chromosomes, show separation of 11 from the remaining 10 chromosomes. As long as the nature of the aggregation of the chromosomes of a whole set is not understood, such subaggregation does not pose new problems.

If genome separation exists, it could form a basis for "somatic reduction" or haploidization. In diploid clones of *Aspergillus nidulans,* Pontecorvo *et al.* ('54) and Pontecorvo and Käfer ('58) showed that somatic variations resulting in mosaic colonies are of two different types. In one, somatic crossing over and segregation, as indicated, produces variant sectors of diploid homozygotes. The other type is the outcome of a process or processes that lead to loss of a whole genome so that the variant sector is haploid. It may be that different mechanisms are responsible for haploidy. Thus successive accidental losses of individual chromosomes may reduce the chromosomes from $2n$ to $1n$. Possibly, somatic haploidization may also be accomplished in a single step, either as the result of chance distribution of chromosomes in abnormal mitoses or as a consequence of genome segregation.

Gläss observed a considerable number of haploid cells in the liver cells of his rats. He is inclined to see in them the consequence of genome segregation. Other ways for segregation of whole genomes to different sister cells by nonrandom processes have been suggested by Huskins and his associates for root tips of different higher plants (Huskins, '48; Huskins and Cheng, '50; Wilson and Cheng, '49; Pätau, '50). Various kinds of somatic reduction have been reported for the mosquito *Culex* (Berger, '38), the radiolarian *Aulacantha* (Grell, '53), and in polyploid species hybrids of cotton (see, e.g., Menzel, '52). Nuclei with an amount of deoxyribonucleic acid characteristic of the haploid nucleus but not necessarily truly haploid have been photometrically recognized in the

otherwise diploid or polyploid somatic tissues of female honeybees (Merriam and Ris, '54). Whatever the mechanism, the origin of haploid from diploid or lower degrees of ploidy from higher ones is an established source of somatic cell variation.

Heteroploidy. The nuclei of somatic cells often contain chromosomes that, in numbers, are not simple multiples of the haploid number. Usually there are more chromosomes than in the diploid, but hypodiploid cells also are found. Hypohaploid numbers are rare, particularly in cells that do not appear to be abnormal. Mechanisms for heteroploidy are well known: (1) nondisjunction of a pair of sister chromosomes in an otherwise normal mitosis, thus leading to inclusion in one daughter nucleus of two instead of one chromosome and of none in the other nucleus; and (2) multipolar mitoses in diploid or polyploid cells as well as other types of abnormal nuclear behavior, including the possible consequences discussed under genome separation. In somatic tissues of the intact organism or in tissue cultures, the various abnormal processes may follow one another in irregular fashion and their results be combined in descendant cells. Such sequence of events may produce cells with the diploid chromosome number, which however are not truly diploid but may have one chromosome represented only once and another three times (Chinese hamster; Tonomura and Yerganian, '56).

Chromosome breakage and rearrangement. The frequency of spontaneous chromosome breakage has been underestimated. In cells with homologous chromosomes that have inverted sequences in relation to each other, crossing over can lead to formation of chromosome bridges followed by breakage, fusion of broken ends, and renewed bridges. *Dissociator* elements in chromosomes may disrupt chromosomal continuity and initiate breakage-fusion-bridge cycles (McClintock, '51). These may not be special situations but rather, ubiquitous. In any case, from less than 1% to 10% or more of cells with one or several visibly broken chromosomes have

been present in tissues from various higher plants (see Swanson, '57, pp. 350–361) and in human tissue cultures (Bender, '57). Nutritional factors, such as deficiencies of calcium or magnesium, as well as irradiation, greatly increase the incidence of chromosome breakage (Steffensen, '53, '55; Bender, '57; see also Puck, '57).

When two or more chromosomes are broken, fusion of broken ends often leads to rearrangement of chromosomes. Depending on the positions of breakage points, reciprocal translocations of segments of similar size may occur between two chromosomes or very unequal parts may be exchanged. An important type of the latter kind of exchange is that resulting from breaks in two essentially rod-shaped chromosomes. If one chromosome is broken near the kinetochore in its long arm and the other in its short arm, the exchange products are one chromosome with two long arms and another with two short arms. Makino and Kanô ('53) have described, in ascites tumors of rats, the presence of various types of large V- and J-shaped chromosomes not represented in the normal chromosome complement.

The breakage-fusion-bridge cycles mentioned earlier are elements of continuous instability in the nuclear constitution of affected cells. These cycles are initiated whenever broken chromosomes fuse in such ways as to form a new chromosome with two kinetochores. In mitosis, the two kinetochores may move to opposite spindle poles. The chromosome bridge between them may break at some more or less randomly selected point, and fusion of the broken ends of the sister chromatids will reconstitute new chromosomes with two kinetochores, and the cycle can start anew.

Aberrant chromosome behavior involving whole chromosome sets, whole chromosomes, deficiencies, duplications, inversions within a single chromosome, and innumerable possibilities of rearrangements between chromosomes must be regarded as an ever-present source of somatic cell variation

Developmental nuclear variations

The appearance of the nucleus varies from tissue to tissue in animals and plants. So does that of the cytoplasm. Whether the property of differentiated tissue cells to reproduce their own kinds, *in vivo* or *in vitro,* depends on nuclear or cytoplasmic properties is, of course, one of the central problems of somatic cell variation. Constancy of differences between cell strains from various tissues over long periods of growth is not a criterion of genetic properties if the term "genetic" is defined in the same way as in experiments in which Mendelian analysis is applicable. The analogy with certain types of microbial variation occurring in asexual cultures, variation proved by recombination analysis to be of genic nature, must not be extended prematurely. There exist after all, alternative schemes of nongenic (though gene-dependent) differentiation involving induced enzyme systems, alternative types of steady states, or the phenomenon of Dauermodifikation.

We might wonder whether those special processes of differentiation, which are regularly accompanied by polyploidy, are the consequences of the multiplication of chromosome sets. Even this suggestive association, however, does not seem to be causal. Ploidy of the already-differentiated trichogen cells in the epidermis of the water bug *Corixa* increases endomitotically with each later molt, parallel with the increase of size of the bristle. In the cabbage butterfly *Pieris* the ploidy of the cells that produce the scales of the wing also increases after these cells have clearly entered the road of their specific differentiation (Lipp, '53, '57).

If polyploidy is not a cause of differentiation, its regular presence in some types of differentiated cells in itself endows these cells with new genetic properties. Some irreversibility in differentiation may thus have a genetic, nucleus-localized basis.

Persistent nuclear variation arising in an orderly way in development has for the first time been demonstrated by the

extraordinary, skillful experiments of serial nuclear transplantation in frogs by Briggs and King (see King and Briggs, '56; Briggs and King, '57). All nuclei of cells from blastulae and early gastrula stages seem to be totipotent as shown by their ability, if transplanted into enucleated eggs, to promote full development. The nuclei taken from the endoderm of late gastrulae are not equivalent any more. After transplantation into enucleated eggs, some still promote normal development throughout but others do so only up to some time after gastrulation, and in still others development is arrested at the gastrula stage. The following experiments show that these different developmental fates were really attributable to nuclear properties. Several blastulae derived from enucleated eggs each with a single transplanted endoderm nucleus were sacrificed, and a number of their nuclei were separately transplanted into new sets of enucleated eggs. The development of these eggs varied in a significant way. Those whose nuclei had come from the same blastula were very similar among themselves, but they often differed greatly from those whose nuclei came from other blastulae. Such serial transplantations repeated three to five times demonstrated that the restriction of potencies to promote development was irreversible under the conditions of the experiments.

The meaning of these facts may become clear after further work. A naive view might have led one to expect that endoderm nuclei, if their development-promoting ability had become restricted, would permit growth of only the relatively late-originating endoderm and of no other germ layer. The experiments, on the contrary, show that development of the early stages of blastula and initial gastrula can still be accomplished. On the other hand, the arrested abnormal embryos displayed pronounced deficiencies of the ectodermal derivatives, in contrast to normal appearance of endoderm tissue. Yet, one wonders whether transplantation of prospective ectoderm tissue from an early gastrula with endoderm-derived nuclei to a normal embryo would not show that the endoderm nuclei can promote development of ectoderm.

The restriction of potentialities in the endoderm nuclei has been established. It is certainly most remarkable that it does not reverse during a series of, maximally, 65 division steps all accomplished in the cytoplasm of successive host eggs that, with nonrestricted nuclei, would have developed all the way. But it does not seem justified at present to conclude that the restriction is attributable to genetic changes in the nuclei. It is conceivable that nuclei from immature germ cells transplanted into enucleated eggs will be shown to promote development in a similarly restricted way, as do nuclei of endoderm cells. If this were so, the restriction would obviously not be of genetic nature since the same nuclei in the germ line would have made unrestricted gametes. It is pure speculation to consider the possible outcome of an unperformed experiment. The speculation is justified, however, if it calls attention to alternative possibilities of interpretation.

Cell variation caused by differential activation of genes

It has been shown that the activity of specific genes can be controlled by special chromosomal elements that become transposed from one locus to another (McClintock, '56). When the controlling element is located close to a specific gene it may fully or partially suppress the gene's function or call forth other functional changes. When the controlling element is removed from the gene, the latter will become able to function, in old or new fashion. McClintock points to observations showing that controlling elements may exert their effect at specific stages of ontogeny. A full theory of development in terms of controlling elements has not yet been presented.

A related peculiar type of cell variation is the basis for variegation in *Drosophila* studied by Schultz and others (see Lewis, '50). When genes normally located in a euchromatic region of the chromosome are brought into proximity to a heterochromatic region, they produce their effect (for example, pigmentation) in some cells and fail to do so in

others. A group of cells derived by mitosis from one cell behave alike, either all or none showing the effects of genic action. Therefore, instead of consisting of a fine pattern of pigmented and nonpigmented cells, the resulting mosaics are composed of larger alternating areas. There is thus considerable persistence or stabilization either of the active or inactive state of the gene in question. The germ cells of such variegated animals always transmit the gene in the variable state, which permits the production of both pigmented and unpigmented cells in the next generation. There are no ways of deciding whether the gene in the germ line never was stabilized or was stabilized for a period but reverted to the variable state.

That somatic cell differentiation in development is linked to gene "activation" has been shown by analyses of gene action in the initiation of differentiation according to fixed patterns (Stern, '56). In *Drosophila,* a gene for presence of bristle organs causes their differentiation to take place at a few specific locations on the thorax. The same gene is present in other cells of the epidermis where, however, no bristles develop. When a mutant allele of the normal gene is substituted, no bristles are formed at the specific locations mentioned. A variety of mosaic individuals have been obtained in each of which some of the cells possessed the dominant normal allele (in heterozygous combination) and others the mutant allele. In such mosaics the appearance of a bristle at its fixed position is solely dependent on the genotype of the cell at that location. This means that, in both a nonmosaic normal and a nonmosaic mutant individual, the places where bristles may be differentiated are predetermined but the genetic competence of the cells located at these singular places is decisive as to the actual differentiation occurring. It would be misleading to say that the cytoplasm of the prospective bristle cell is the agent that differs from the cytoplasm in the surrounding cells. The regional singularity (unknown as to its molecular nature) of the specific location is the result of its relative position in the epidermis. The property that distinguishes the

specific cell from the rest affects it as an as yet unanalyzed whole. All that is known is that an effect of the normal gene is evoked in this cell only and not in others. One may recall Wigglesworth's mode ('45) of viewing an organism as a chemical continuum that provides for the unity of the organism.

The term "activation" of gene as used here and elsewhere is not a fully appropriate one. At a molecular level the normal gene may be active everywhere. Conceivably, its bristle-promoting function may truly be activated only where the effect becomes visible; more likely, the function may be present in all cells but at a level that remains below the threshold of a differentiation effect. Cytological changes, ordered in time and according to organ, in the appearance of specific bands of the giant salivary gland chromosomes of flies seem to be visible evidence for an ebb and flow in the activity of genic loci (Beermann, '52; Mechelke, '53; Breuer and Pavan, '53, '55).

THE EXPRESSION OF SOMATIC NUCLEAR VARIATION

When a somatic cell acquires a nucleus genetically different from that of its mother cell, the new genotype may or may not be apparent in a new phenotype. Whenever a new phenotype appears in such a single cell, it constitutes evidence of genic action through the membrane of the interphase nucleus.

Although it seems obvious that genes must act through the nuclear membrane, direct evidence is still very limited. Unspecifically, radioisotope experiments on nondividing amebae have shown that the nucleus is involved in P^{32} uptake and in protein synthesis (see Mazia and Hirshfield, '50; Mazia and Prescott, '55). Specific effects of interphase nuclei, distinguished by complex and undefined genetic differences have been demonstrated by Hämmerling ('53) by means of exchanges of the nuclei between *Acetabularia mediterranea* and *A. wettsteinii.* A few still more specific examples of immediate expression of nuclear variation in terms of individual genes collected from the literature 20 years ago (Stern, '38) have

remained rather isolated. This does not mean that genes act only rarely through the nuclear membrane but rather that the study of single cells directly after they have acquired a new genotype is not often possible.

Cells or cell patches whose genotype varies from that of their surroundings will fully express that fact phenotypically only when each cell is autonomous, i.e., independent of surrounding cells and their gene-dependent products. The phenotypes of even small patches of tissue in the petals of *Delphinium* (Demerec, '31) or of the shape or color of bristles on *Drosophila* (Sturtevant, '32) as well as of many other genetic mosaics are proofs of autonomous, intracellar expression of variant genetic constitution acquired by mutation, somatic crossing over, or otherwise. In mammals, including man, autonomy in expression of somatic cell variants is exemplified by specimens whose blood-forming cells are of two types — that of the individual's own genotype and that of a twin sib, some of whose cells had been transferred *in utero*. The red blood cells of such mosaic individuals may be of two different antigenic types, each genotype of the erythroblasts expressing itself autonomously in the characteristic of the erythrocytes.

Absence of autonomy likewise is known. In *Drosophila,* a patch of eye tissue or a whole eye with the *vermilion* genotype on a non-*vermilion* fly usually does not possess the *vermilion* phenotype (Sturtevant, '20). This is now well understood, since it is known that the cells with a non-*vermilion* genotype provide kynurenine (which is necessary for non-*vermilion* eye color) for the cells with the *vermilion* genotype who themselves cannot form this derivative of tryptophan (Butenandt *et al.*, '40). Intermediate situations also exist in which a genetically variant tissue can express itself phenotypically to some degree but can also exhibit some influence of the neighboring tissue [*Delphinium*, Demerec ('31); *Drosophila,* Hannah ('53)].

In bacterial cultures, a genetic change often expresses itself only after a number of divisions. Such phenotypic lag has

been attributed to various causes. One of them is the presence of some material in the cell that was elaborated during the existence of the original genotype and that first may have to be exhausted. In ciliates, specific antigenic properties and mating types once established may be propagated for many divisions. They are independent, to a degree, of the genotype that may make these phenotypes possible but is not uniquely related to them (Sonneborn, '50; Beale, '52; Nanney, '57).

In this connection, the phenomenon of maternal effect observed in higher organisms is relevant. Here, the genotype of the mother impresses properties on the egg that become expressed phenotypically in the offspring, regardless of its own genotype. Sometimes, the maternal influence is exerted so early in the embryology of the young that its persistence in later life is a simple consequence of the early effect. A well-known example is the inherited asymmetry, dextral or sinistral, of the snail *Lymnaea* that is fixed by the maternally controlled position of the first-cleavage spindle. In other organisms, a maternal effect may induce an abnormality of the gonads of the offspring, an event that becomes apparent only late in development (e.g., *Drosophila subobscura,* Spurway, '48). One further instructive example of a late-acting maternal effect concerns the position of the symmetry bands on the wing of the meal moth *Ephestia* (Kühn and Henke, '36; see Kühn, '55). The position of the band is controlled by specific genes, and its development in the pupal wing partly depends on the genotype of the mother.

Differentiation of a given tissue in development may depend on influences emanating from another tissue. Once accomplished, differentiation usually is irreversible. This seems to relegate to idle fancy the idea of testing the genetic constitution of fully differentiated cells by means of ordinary Mendelian hybridization methods. But have we not overlooked something? Is not an egg and perhaps even more a sperm a most highly differentiated cell? The differentiation of these cells specifically fits them for the genetic analysis we

require. It shows their totipotency, their possession of a complete genetic endowment. Is it necessary to assume that differentiation of the germ cells is any different from that of somatic cells?

The germ cells provide one more type of phenomenon to prove that a specific differentiation is not dependent on but superimposed upon a constant genotype. Many years ago, Witschi ('14) propounded the theory that the differentiation of egg and sperm in amphibian gonads, and in the gonads of vertebrates in general, is not dependent on the sexual genotype of the cells themselves but on the type of nongerminal gonadal tissue in which they find themselves. Transplantation experiments in the salamanders *Ambystoma tigrinum* and *A. mexicanum* by Humphrey ('45, '57) as well as hormonal treatments in the medaka fish *Oryzias latipes* (Yamamoto, '55), and in the newt *Pleurodeles waltlii* (Gallien, '54) have brilliantly supported the main tenor of Witschi's theory. Genetically female cells could be made to enter the path of differentiation leading to functional sperm and genetically male cells into differentiation of functional eggs. Breeding tests showed that the sperm came from cells that had retained the female genotype, and the eggs came from cells with the original male genotype. These experiments demonstrate the persistence of phenotypes imposed "contrary" to the genotypes. They also furnish unique evidence for the view that differentiation can be an epiphenomenon, however incisive, of an invariant genotype.

THE SELECTIVE PROPERTIES OF SOMATIC CELL VARIANTS

Variations that arise in the germ cells of multicellular organisms may be exposed to selective forces early, when the fertilized egg begins its development or late, when the body is built up of equally variant cells. Striking chromosome abnormalities may result in death of early embryos, as has been shown for *Drosophila,* and as a consequence of radiation for mice and rats. Variants that are not eliminated as rigorously

as these may, of course, possess all different degrees of selective attributes.

The selective value of cell variants arising within the soma of an individual may also be a direct function of the new constitution or it may be related to an interaction between cells with the new variant genotype and with the original one. Some genotypes lethal to the individual as a whole, if it begins life with them, are lethal even to individual cells somatically segregated out on a nonlethal main body (*Drosophila,* Demerec, '34). Other genotypes equally lethal to the whole individual, if present in the zygote, permit viability of cells and tissues either if removed from the lethal influence of the whole or if supported by a nonlethal cellular environment (mouse, Ephrussi, '35).

Selection against somatic cell variants may thus be less rigorous than against germinal ones. A genotype that cannot support the whole complexity of ontogeny may well be able to support the lesser, though still formidable, complexity of cellular physiology. This is true to a much greater degree than would have been predicted only a few years ago. Proof for it is provided by the survival and the tragically exuberant growth of mammalian cells that possess the most deviant chromosome numbers and constitutions, as seen in ascites and other tumors as well as in tissue cultures.

During the evolution of new systematic categories, genetic newness once arisen has to be adapted to long-range survival by selection of suitable genetic backgrounds. The same process must occur in the history of somatically variant strains. The unbalance, for instance, of an initial chromosome aberration — provided that it is at all compatible with survival and reproduction of the cell — may become diminished by a suitable selection of additional genetic variations arising somatically. A striking example is provided by the history of the diploid gametophyte of the moss *Bryum caespiticium* experimentally produced by Wettstein ('37). At first these plants showed gigas characters including abnormally large cell size as compared to the normal, haploid form. But in the course

of 11 years of vegetative reproduction and without change in the diploidy of its nuclei, the cell size had gradually decreased from 34,200 to 18,600 μ, the latter value not much larger than that of the original haploid plant with a volume of 16,900 μ. Simultaneously and probably as a consequence of this normalization of cell size, other ill-adapted phenotypic traits such as the gigas character of the plants and their near sterility had gradually disappeared. These observations on mosses undoubtedly reveal models for similar events occurring in mammalian tissue cultures.

The role of the nucleus in somatic cell variation is similar to its role in variation of whole multicellular individuals. In this recognition we agree with Bateson ('26), who in his posthumous paper entitled "Segregation," stated, "I am tempted to regard Mendelian segregation in general as a process not essentially different from that which we see working in the mosaics." Today, we are inclined to turn around and expand Bateson's sentence. Somatic cell variation depends on a variety of processes that are essentially not different from those working in Mendelian segregation, normal and exceptional. These processes provide the basis for the understanding of large areas of somatic cell variation.

OPEN DISCUSSION

Boyes [2]: Professor Stern has pointed out the importance of somatic crossing over. I have examined hundreds of somatic cells in the Diptera and should like to emphasize that in prophase their chromosomes are not just side by side, as one usually sees them illustrated, but are actually twisted around each other. Seeing this is rather convincing evidence that this exchange can take place. Another point is that even in the tetraploid nucleus there is somatic pairing.

Barigozzi [3]: I was very much interested in hearing of all the different mechanisms Dr. Stern proposed to explain

[2] J. W. Boyes, McGill University.
[3] C. Barigozzi, University of Milan.

changes in chromosome numbers in somatic cells. It seems to me that he assumed that all chromosomes are multiplying at the same time, which is certainly difficult to disprove. But it is also possible that in some cases chromosomes do not all divide at the same time during resting stage. I observed this several years ago, especially in cells of the silkworm and insects.

Those cells, especially those of the silk glands of the silkworm, show very large branched nuclei. During formation of these nuclei, it can be shown that the different chromosomes are dividing at different times, according to their different sites within the same nucleus. Some chromosomes show a metaphasic structure while others are in a resting stage structure. Branching of the nucleus therefore occurs step by step.

From a given point of the nuclear surface, something projects, like a bud, and the chromosome number also increases step by step. It might be that, when the nucleus is undergoing such a chromosome number increase at, for instance, the diploid stage, an equilibrium of the genotype is also reached, and there is no further growth stimulus. Certainly with such a procedure, the greatest different numbers of chromosomes can be produced. I must say that my observations have not met with wide approval among cytologists. I know, for instance, that Geitler does not believe this, but I still maintain my view, which permits an explanation of all kinds of different numbers and all the different stages between diploidy, tetraploidy, and so on.

Stern: Those are very interesting observations. There have been a few fragmentary ones of this type. Jack Schultz, for instance, has thought that in the nuclei of the nurse cells of *Drosophila* the Y chromosome does not reproduce at the same rate as the other chromosomes. Also, endomitoses have been described in which there is prophase in one part of the nucleus and metaphase in another part. Even if Dr. Geitler does not support you, I think these are things we have to look for.

HAUSCHKA[4]: There are several other possibilities for interpreting apparent genome separation. For instance, Wilson described arrested anaphases in liver. Failure of cytokinesis is perhaps owing to spatial confinement, and results in cells with *two* diploid nuclei. If, during the next mitotic cycle, these nuclei form two diploid metaphases in one cytosome, this might look as though the genomes of a tetraploid cell were separating.

Second, cells with lobated nuclei are very common in lymphosarcomas and in mast-cell tumors. From the studies of Levan and others, we know that the individual nuclear lobes or separate pockets undergo synchronized prophases. In squash preparations, one obtains various counts of chromosomes in the individual lobes, or micronuclei, some of which may appear to be haploid, and they may add up to higher multiples of n per cell, again suggesting genome separation of an orderly nature.

Third, cell fusion without nuclear fusion seems to occur in both tissue culture and regenerating liver. If it is assumed that a tetraploid cell fuses with a diploid one, during the next mitotic cycle the composite cell may have two nuclei with different multiples of n. I think such phenomena could be misinterpreted as pseudomeiotic happenings or genome separation in somatic cells.

STERN: To that I might add that I saw a film by the Lettrés, from Germany, in which fusion of two cells resulting in a binucleate cell could be followed under the phase microscope.

GELFANT[5]: There is one major chemical difference in the similarity between the sperm cell nucleus and the somatic cell nucleus. The sperm nucleus generally does not contain nonhistone protein but the somatic cell nucleus does. Of course the nonhistone protein fraction varies considerably in somatic cell nuclei in relation to the metabolic activity of the cell. Do you consider the sperm cell nucleus and somaitc cell nucleus similar or dissimilar?

[4] T. S. Hauschka, Roswell Park Memorial Institute.
[5] Seymour Gelfant, Syracuse University.

STERN: Before it is a sperm nucleus, it is a spermatogonial nucleus, and at that time it is a differentiated cell like others.

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THE CYTOPLASM AND SOMATIC CELL VARIATION

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I was asked to speak on "The Cytoplasm and Somatic Cell Variation" and I apologize for not adhering strictly to this title. To a large extent this is because a Conference on "Extrachromosomal Heredity," recently held at Gif, greatly influenced my thinking on this subject. The views I am going to express here probably do not represent the cross section of opinion at the Gif Conference but they bear its mark. In particular, they are influenced by some arguments of Dr. D. L. Nanney, in part unpublished (see however Nanney, '57) to whom I therefore owe an apology for using them, explicitly or implicitly. One of Nanney's points is that the usual classification of genetic mechanisms on what he calls "the geographical basis" may be very misleading. Nanney's arguments appeared to me so important that, in preparing this paper, I was unable to limit myself to the consideration of the cytoplasm alone.

Ever since I took up the genetic tool, the problem of embryonic differentiation has been in the back of my mind. All along, one of my persistent claims has been that changes during development do lie within the province of genetics, and I do take the title of this symposium as a sign that it is now generally felt that variations in development do pose a problem to the geneticist. I therefore have the rather comforting feeling that the time may be over when, to pull down a geneticist, another geneticist would simply have to say, "Well, he started

as an embryologist!" However, when treated this way, I used to find consolation in the fact that I was in good company: T. H. Morgan, for one, started as an embryologist, too.

And this reminds me of a conversation I had with Dr. Morgan in the summer of 1934 at Woods Hole. One day he told me that his book "Embryology and Genetics" had just come off the press. When I told him how anxious I was to read it, he offered to give me the copy he had on his desk, provided that I promised to read it and to give him my frank opinion about it. I accepted, and a few days later went in to report. I said I found the book very interesting, but I thought that the title was misleading because he did not try to bridge the gap between embryology and genetics as he had promised in the title. Morgan looked at me with a smile and said, "You think the title is misleading! What is the title?" "Embryology and Genetics," I said. "Well," he asked, "is not there some embryology and some genetics?"

This shows how polarized I was on the gap between embryology and genetics, and how anxiously I was waiting for somebody to bridge it. And so was Morgan, for he stated the problem on the very first pages of his book. In fact, he did end up with a most interesting suggestion, which I overlooked when I talked to him, and I still do not know whether his evasive answer to my criticism was owing to his tactfulness and modesty, or was because he regarded the suggestion he made as rather too speculative. The concluding lines of his book, which contain this suggestion, show how close it was to what was discovered some 20 years later.

"As I have already pointed out, there is an interesting problem concerning the possible interaction between the chromatin of the cells and the protoplasm during development. The visible differentiation of the embryonic cells takes place in the protoplasm. The most common genetic assumption is that the genes remain the same throughout this time. It is, however, conceivable that the genes also are building up more and more, or are changing in some way, as development pro-

ceeds in response to that part of the protoplasm in which they come to lie, and that these changes have a reciprocal influence on the protoplasm. It may be objected that this view is incompatible with the evidence that by changing the location of cells, as in grafting experiments and in regeneration, the cells may come to differentiate in another direction. But the objection is not so serious as it may appear if the basic constitution of the gene remains always the same, the postulated additions or changes in the genes being of the same order as those that take place in the protoplasm. If the latter can change its differentiation in a new environment without losing its fundamental properties, why may not the genes also? This question is clearly beyond the range of present evidence, but as a possibility it need not be rejected. The answer, for or against such an assumption, will have to wait until evidence can be obtained from experimental investigation'' (Morgan, '34, p. 234).

From what experimental investigation could we hope to obtain the required evidence?

I shall not go to the excess of that great British friend of ours who once told me, ''When I want to read a good book, I write it,'' but I shall quote a few lines of my own to show how I saw the situation in 1952:

''. . . what is needed is direct genetic analysis of somatic cells, for the assumed functional equivalence of [nuclei of] irreversibly differentiated somatic cells, however plausible, is only an hypothesis. Crosses between such cells being impossible, only nuclear transplantation from one somatic cell to another, or grafting of fragments of cytoplasm, could provide the required information; such experiments however will have to await the development of adequate technical devices. In the meantime, the closest approximation to the evidence we would like to have is provided by the study of lower forms which propagate by vegetative reproduction and possess no isolated germ line'' (Ephrussi, '53, p. 5).

Prompted by these thoughts, I had already joined the ranks of the microbial geneticists and was wandering on the back-

roads, toward my goal when I wrote these lines, and R. Briggs and T. J. King were already fighting their way to the highway of nuclear transplantation. What has happened since is well known. Curt Stern (this symposium) spoke of their beautiful work and spectacular results on real embryos, not just models. Were then the efforts of the microbial geneticists wasted in so far as the embryological problem is concerned? This question must be answered, I feel, by a most emphatic "**No**," and I shall give several reasons for thinking so.

First, microbial genetics has provided a new methodology for the study of somatic cell lines. Second, microbial genetics has also provided several new techniques for genetic analysis of somatic cell lines, namely transformation, transduction, and mitotic recombination. Third, microbial genetics has uncovered a number of new mechanisms of inherited variation, none of which may be rejected on *a priori* grounds as possible agents of somatic variation in higher forms. Moreover, the discovery of these new mechanisms has occasioned much theoretical discussion, which has resulted in clarification of the significance of some concepts fundamental to both embryology and genetics, such as the concept of self-reproduction (see Lederberg, '52; Pollock, '53; Lederberg and Lederberg, '56).

I shall not elaborate on the first point. The essence of the new methodology and its fruitfulness will, I am sure, become clearly apparent to those not working in the field from the papers by Marguerite Vogt and T. T. Puck (this symposium) on *in vitro* culture of somatic cells. Nor shall I discuss the second — the merits of transduction, transformation, and mitotic recombination — either for their intrinsic value or as possible techniques in the analysis of somatic cell variation. All I wish to say on this point is that, in my opinion, the nature of somatic cell variation, including the nature of the nuclear changes observed by King and Briggs ('56), will not be cleared up without resort to these devices.

But I should like to mention briefly some of the unorthodox types of cell variation discovered in microorganisms (along,

of course, with the purely Mendelian ones). Here I cannot give anything approaching a complete list. Nor can I give a general picture, for the only feature common to all these cases is that they concern stable differences between cell lines, inherited in a way not conforming to the rules of Mendelian inheritance: the seat of the changes is therefore said to be "extrachromosomal" or "cytoplasmic." Aside from that, they seem to follow no common rule: there seem to be almost as many patterns of manifestation, variation, and transmission as there are individual cases; and there seems to be no unity of biological purpose. There is no question that a cursory inspection leaves one with the disastrous impression of a collection of biological oddities.

Can we try a classification on the basis of probable mechanisms? Two general types of mechanisms have been suggested to account for cytoplasmic variation and inheritance. The first is based on the assumption of cytoplasmic self-reproducing particles, i.e., particles carrying within them structural information. The second type of mechanism is the so-called steady-state mechanism, to which Sewall Wright and L. von Bertalanffi first called attention and that, as Max Delbrück ('49) pointed out, is capable of perpetuating differences arising without change in the intrinsic properties of the system, i.e., without changes in structural information. I may add that the reality of persistent variations of this type in biological systems has been demonstrated by Melvin Cohn ('57) in the case of the inducible permeation factors in *Escherichia coli.* Furthermore, Novick and Weiner ('57) showed that the transition from the fully induced to the uninduced state bears a close resemblance to mutation. I cannot stop to give details of the experiments mentioned. Nor shall I insist at this point on the very real difficulty of the operational distinction between the two types of mechanisms. This has been aptly discussed by several authors (in particular, Lederberg, '52; Pollock, '53), and indeed, it has been suggested that possibly there is no real difference between the two systems (Lederberg and

Lederberg, '56). Whatever the deeper significance of the apparent differences, I feel that, in some selected cases, the weight of evidence clearly favors one type of interpretation rather than the other.

Since the chief distinction between the two mechanisms resides in the fact that only in the former are stable modifications of cellular specificity based on changes of structural information, let us consider the few cases of cytoplasmic transmission where the source of information is more or less clearly established.

The case I shall mention first is that of T. M. Sonneborn's kappa particles in *Paramecium;* this is certainly the one case where extranuclear localization of the genetic information is established beyond doubt, for these particles occur in several mutant forms. The fact that kappa particles do contain deoxyribonucleic acid suffices by itself to convince some people that they are truly genetic particles, and others that they are not truly cytoplasmic (i.e., that they are parasites). Although I do not agree with either argument, I agree with both conclusions.

Next, I shall briefly mention plastids. Historically, they represent, I believe, the first case of alleged complete reproductive autonomy with respect to the nucleus. By now, many nuclear influences on chloroplasts have been discovered, including effects on their mutability (see Rhoades, '55). Doubts have been expressed about their ability to mutate and, hence, about whether they really contain within them structural information. Rhoades (loc. cit.) considers that convincing proof of plastid mutations has been provided by M. W. Woods and H. G. Du Buy's observations on *Nepeta,* and I should like to add that, in my opinion, de Deken-Grenson and Messin's study ('58) on *Euglena* dispels my doubts about the location of the information, at least in this case. These authors have shown that a streptomycin-treated *Euglena* can form new chloroplasts only so long as it still contains at least one such element. Irreversibility of loss is of course not a foolproof argument,

but, in a complex structure like a chloroplast, I consider it rather convincing.

Chloroplasts can, however, hardly be regarded as typical components of mammalian cells, and I brought them up here only because, aside from providing the extreme example of autonomy, they are, in the eyes of many cytologists, related to the ubiquitous mitochondria.

The reproductive autonomy and mutability of mitochondria have been suggested by several authors (Rhoades, '50; Caspari, '56) and, according to some reviewers, by me. My actual claim will be clear from the following:

In yeast, a frequently occurring and easily inducible mutation results in the permanent inability of the cells to respire. The mutation apparently involves no genotypic change: this was first shown by the non-Mendelian results of crosses between normal and respiration-deficient yeasts. Cytoplasmic transmission was even more convincingly indicated by Wright and Lederberg's study ('57) of the vegetative progeny of heterokaryons, which clearly showed the absence of a correlation between respiratory phenotype and parental nuclear constitution.

The irreversibility of the respiratory defect, the pattern of appearance of the mutants — the fact that the induction of the mutation is subordinated to cell division — led to the assumption that normal yeast contains a particulate self-reproducing factor that is lost or inactivated in the mutants.

The respiratory defect of the mutants is attributable to the simultaneous absence of several respiratory enzymes (cytochrome oxidase, succinic acid, reduced diphosphopyridine nucleotide, α-glycerophosphate, cytochrome c reductases, cytochromes a and b), all known to be carried, in the normal cell, by the mitochondria (Slonimski, '53). This led to the suggestion (not claim) that the cytoplasmic factor, postulated on genetic grounds, may be identical with the mitochondria. Today, however, there is good cytological evidence that the mutant cells do contain mitochondria (Yotsuyanagi, '55).

Therefore, if the cytoplasmic factor has anything to do *directly* with mitochondria, it is either not lost but mutated, or else it represents just one of several morphologically indistinguishable but biochemically distinct types of mitochondria.

The hypothesis that the cytoplasmic factor and the mitochondria are the same remains attractive because it makes it easier for us to visualize the simultaneous loss or inactivation of a whole enzyme complex.

Where in this case is the genetic information located? There are three reasons that make me think it must be located within the hypothetical particles themselves: (1) the defect is irreversible; (2) in the presence of a certain recessive gene, the cytoplasmic factor goes on propagating itself, although it is apparently physiologically inactive; and (3) it seems improbable that a chromosomal change would simultaneously block the synthesis of so many different enzymes. None of these arguments is, of course, watertight, but, taken together, they seem to me to carry considerable weight.

I shall omit several cases of cytoplasmic inheritance ascribed or ascribable to viruses (just because I prefer to avoid a philosophical discussion of "normal" and "abnormal") and shall turn to the cases where the structural information is clearly in the nucleus. Let us consider first the antigenic types of *Paramecium,* beautifully worked out by T. M. Sonneborn and G. H. Beale (see Beale, '54). Here, the situation can be stated rather simply without going into details. Individuals of each variety of *Paramecium* can assume a definite number of serotypes characterized by a corresponding number of surface antigens. The number and nature of the different antigens a given variety can produce are strictly specified by an equivalent number of nonallelic genes. Antigenic types are clonally propagated, but massive changes to another type can be induced in a clone by a number of environmental shocks. The new antigenic phenotype, induced by the shock, is thereafter as stable in reproduction as was the previous one.

The nuclear constitutions of two individuals of different antigenic types can be equalized by conjugation, which normally involves no cytoplasmic exchange. When this is done, the clones formed by each of the mates exhibit the antigenic type of the exconjugant from which they are derived. When, exceptionally, conjugation is accompanied by cytoplasmic, exchange, the serotype of one of the conjugants (and of the clone derived from it) is switched to that of the mate.

The transmission of serotypes is thus purely cytoplasmic, even though the structural information is in the nucleus. The role of the cytoplasm is that of a selecting device: it selects which genes will come to expression. This selecting capacity itself is, however, subject to changes, resulting in a number of "cytoplasmic states" that, although never irreversible and always subject to external stimuli, have a very strong tendency to self-perpetuation.

The physical basis of these cytoplasmic states is of course unknown; but their multiplicity, mutual exclusion, and constant reversibility are among the facts that prompted Sonneborn (see in particular Sonneborn, '51) to abandon in this case the particle hypothesis and to assign greater probability to interpretations of the steady-state type. What particular form of this hypothesis will finally prove to be correct is immaterial right now. The essential point is that we have here self-perpetuating cytoplasmic conditions obviously not based on changes of structural information.

Before pushing the argument to its logical conclusion, let us consider the mating type system of *Paramecium,* where the respective roles and interactions of nucleus and cytoplasm are better understood (cf. Beale, '54; Nanney, '57). The system has the additional advantage that here the determination of essentially the same trait follows entirely different patterns in the two groups of varieties of *Paramecium,* A and B. In both groups, individuals of identical genotype are bipotential and can exhibit each of two alternative mating types.

In *Paramecium* of group A, variety 1, mating type determination is strictly nuclear and, more precisely, karyonidal. After conjugation, during which two new macronuclei are formed in each of the mates, each karyonide (i.e., clone containing the descendants of one of the new macronuclei) is pure for one of the mating types, which may or may not be the same as that of the sister karyonide, even though the two macronuclei are formed in the same cytoplasm. Clearly, then, mating type depends on the state of the nucleus, and no cytoplasmic influence is detectable.

In variety 4 of group B, the situation is very different (and, in fact, very similar to that of the serotypes). After animals of the two mating types VII and VIII have undergone normal conjugation, each of the exconjugants gives rise to two karyonides of its own mating type. There is clearly an effect of the cytoplasm and this is confirmed by the difference in results. when conjugation is accompanied by cytoplasmic exchange. In this case, the mating type of one of the mates is changed to that of the other. Depending on the temperature, both become either VII or VIII.

This, however, occurs only when, as in normal conjugation, the old macronuclei degenerate and new ones are formed from the synkaryon. However, a trick can be used to modify this process in such a way that part of the progeny of one exconjugant receives a new macronucleus, while the other regenerates a marconucleus from a piece of the old one. It then appears that the change of mating type occurs only where a new macronucleus has developed, and an individual carrying the old macronucleus cannot change its mating type. Thus the action of the cytoplasm is via the macronucleus: mating type depends on the state of the macronucleus, but the state of a macronucleus is determined during its differentiation by the state of the cytoplasm.

The last question to be answered is whether these cytoplasmic states are really autonomous or, on the contrary, ultimately controlled by the nucleus. The two types of individuals just

referred to (with new and old macronuclei, respectively) were produced by a single animal and therefore must have had, initially, the same cytoplasm. The question now is whether the state of the cytoplasm remained unchanged during multiplication. This was tested by inducing autogamy in the two sorts of individuals; i.e., the formation of new somatic nuclei. The state of the cytoplasm was then found to be always in unison with the state of the nucleus in the presence of which it was formed (Sonneborn, '54). Thus, although the state of the cytoplasm directs the differentiation of a new macronucleus, it is, itself, determined by the existing macronucleus. In other words, there is a completely circular relation that, considered alone, does not permit a decision of what is primary and what secondary. However, the system of mating type determination in group B is so similar to that of the serotype system that there is little doubt that here, too, the source of structural information is in the nucleus; and the absence of cytoplasmic effect in group A supports this interpretation.

Mating types in both groups A and B thus seem to be under the control of the functional differentiation of the macronucleus, and the difference between the two patterns (i.e., the presence or absence of a cytoplasmic influence) may be owing to the "spilling" (to use Nanney's picturesque expression) or an inhibitor of one of the mating types into the cytoplasm.

What kind of changes the macronuclei undergo in their differentiation is not known, but they may be very similar to those discovered by J. Lederberg in his study of phase variation in *Salmonella* (even though the macronuclear changes seem to be irreversible, whereas those studied by Lederberg are reversible — see Nanney and Caughey, '55). The flagella of each strain of this organism occur in two alternative antigenic phases, rather stable in clonal multiplication but occasionally shifting from one to the other. Transduction analysis has shown that the two phases correspond to two nonallelic loci, H_1 and H_2. Phase variation is caused by changes of the functional state of the H_2 locus. "When this gene is in

the active or epistatic state, its allele is phenotypically expressed, and the H_1 factor is functionally suppressed. Alternatively, when the H_2 gene is in the inactive or hypostatic state, the H_1 factor is expressed. While the H_1 and H_2 factors are thus functionally complementary, the H_1 factor does not vary in its *heritable* state, and its activity depends on the state of the H_2 locus. Therefore, when an inactive H_1 is transduced, from a donor in phase 2 where H_2 was epistatic, to a recipient in phase 1 wherein H_2 is hypostatic, the H_1 factor can be immediately expressed. The experiments have thus indicated that the 'state' of the H_2 factor is inherited as such with the transduction of H_2 specificity, while the functional state of H_1 is not'' (Lederberg and Iino, '56, p. 753).

Again, I shall not go into the details but shall proceed directly to the suggested mechanism that will remind you of Morgan's suggestion. I quote, ''. . . epistatis by H_2 might depend on local saturation by the immediate products of its action, a steady state or feedback condition which would depend in turn on the functioning of the cytoplasm and the competitive inefficiency of a stripped H_1 locus'' (Lederberg and Iino, '56, p. 753).

Nobody here, I am sure, was surprised that Lederberg at once realized both the similarity of the changes in functional states of genes that he observed in *Salmonella* with those involved in the mating type determination and their possible relevance to the problems of development and differentiation. The only thing that surprises me is that Nanney coined a new name for this kind of phenomenon before Lederberg did. At the Gif Conference, Nanney proposed to call ''epigenetic'' all mechanisms that regulate the expression of genetic potentialities, in contradistinction to the truly genetic mechanisms that regulate the maintenance of the structural information. (Nanney really suggested ''paragenetic,'' but shifted to ''epigenetic'' after G. Pontecorvo told him of the troubles he got into with a professor of Greek, owing to his ''parasexuality.'') I would personally like to add the suggestion that the

epigenetic mechanisms must be distinguished from the more trivial, immediately reversible phenotypic mechanisms.

Using this terminology, I may now summarize by saying that, in some of the described cases, cytoplasmic transmission depends on truly genetic mechanisms (i.e., the transmission of particles carrying their own structural information), and in some others on epigenetic mechanisms involving functional states of the nucleus.

Which of the described cases are relevant to the problem of normal somatic differentiation? Kappa particles may be discarded because they probably are parasites, and chloroplasts may be ignored on the ground that they are confined to plants. The only other case of self-reproducing particles I mentioned is that of the "respiratory particles" and mitochondria, but as stated, neither the particulateness of the particles nor their identity with mitochondria has been proved. It remains possible, therefore, that we are dealing here, after all, with an epigenetic phenomenon. In fact, I could cite a couple of points rather favorable to such a view, but I shall neither do this nor insist on the opposite view (which I still consider more probable) because I wish to emphasize the remaining cases.

These remaining cases are all examples of nuclear epigenetic mechanisms, where cytoplasmic transmission only masks nuclear determinism. All of them concern, of course, the ciliates, organisms with a very special cellular organization. But I do not believe this is a reason for disregarding the mechanisms their study revealed: we do not disregard the antiproton as a general phenomenon just because it took a very special machine to discover it. And I do believe that the study of ciliates has revealed some fundamental mechanisms and taught some lessons important for those who are going to use the genetic approach to somatic cell variation in higher organisms. These lessons, in my opinion, come under three headings: (1) what to expect, (2) what not to expect, and (3) what to avoid.

What we may expect to find in the genetic study of somatic cell variation are, first of all, cytoplasmic phenomena. I think

they are likely to be encountered just because the cells to be submitted for the first time to genetic analysis are not germ cells but somatic cells that actually have undergone differentiation. Second, and for the same reason, I think we may expect to find somatic variation to be based not only on classical genetic mechanisms but on epigenetic mechanisms as well. Third, we may expect great difficulties in distinguishing genetic from epigenetic and cytoplasmic from nuclear variation.

I have already mentioned the theoretical difficulties, but should like to add a word on the difficulty of finding adequate criteria for distinguishing some of the changes I have described. For example, according to Lederberg, phase variation in *Salmonella* (which I classify as an epigenetic change) is limited to a single pair of alternatives, and this suffices to distinguish it from ordinary mutation because "we observe no mutation in antigenic specificity, only a choice of which of the two alternatives will be expressed" (Lederberg, and Lederberg, '56, p. 114). Now, is this a sufficient criterion for the diagnosis of epigenetic changes? It would seem to apply to the mating types of *Paramecium,* but then the variations in group B present many similarities to the variations of antigenic types that are not restricted to a single choice!

On the other hand, the changes in genes of yeast heterozygotes that Roman ('56) does not want to call "gene conversions," are restricted in a given heterozygote, as he has shown, to a single pair of alternatives: one allele changes to that other that is present in the homologous chromosome. Yet it seems probable that he is dealing, not with epigenetic, but with truly genetic changes of some sort.

So I doubt that Lederberg's criterion, sufficient to distinguish some changes from the classical mutations, is by itself sufficient in all cases. Furthermore, I think that it probably throws into one bag a number of different mechanisms, epigenetic or not.

Epigenetic mechanisms are generally very sensitive to environmental stimuli, but this also does not help us to distin-

guish them with certainty from cytoplasmic particulate mechanisms, for these, too, are very sensitive to the environment.

I shall not go on listing the difficulties, because I have said enough to show that the one thing we should not expect is — an easy job.

This brings me to the last lesson — what to avoid. What we should try to avoid above all is, of course, confusion, and the first thing to do to avoid it, is to refrain from using the same name for different things. I think that the adoption of the terms "genetic" and "epigenetic" may serve a useful purpose. For my part I adopt them because they more clearly delimit the notion of truly genetic change. This is a concession on my part, and many of my geneticist friends will, I am sure, enjoy this shift of my stand. Unfortunately, I must remind them that, as a corollary, we must admit that not everything that is inherited is genetic.

The second thing we should avoid is basing the distinction of mechanisms on nuclear or cytoplasmic localization. In my opinion this has been a major source of confusion in the past, and it is not going to be so easy to avoid it in the future because we have all been trained to regard the problem of differentiation as a nucleus/cytoplasm dilemma.

The third and last thing we should avoid is taking for granted that all cytoplasmic effects are going to turn out to be epigenetic and ultimately nuclear.

I have deliberately refrained from consideration of cytoplasmic organization and structures, and I make strong reservations on their ultimate nuclear control. You will not be surprised that, in this class of structures, I include the mitochondria.

OPEN DISCUSSION

Luria[1]: I should like to add another category to the two — nuclear and cytoplasmic changes — discussed by Dr. Stern and Dr. Ephrussi as the possible causes of somatic cell variation. This category is infection. Here belong the phe-

[1] S. E. Luria, University of Illinois.

enomena of viral infections, in which fragments of genetic material controlling the properties of the cells can migrate from one cell to another. Many of the cellular changes observed in differentiation and in somatic variation are duplicated by viral infections. A few examples will be mentioned.

In bacteria, work on transformation and transduction has shown that every genetic determinant, or group of determinants, of a bacterial cell is transmissible from cell to cell, if the proper tool is available to carry it — a biochemist or a virus! The newly entered genetic determinant, once it has penetrated a cell, becomes part of the heredity of the cell. Today, in bacteria, it is hard to distinguish between a viral and a nonviral element of genetic material. Certain bacterial viruses control not only the synthesis of viral deoxyribonucleic acid (DNA) and viral proteins, but also specific cellular properties, such as somatic antigens.

These viral fragments of genetic material, which function as determinants of cell function, can lose their viral properties by mutation, becoming defective proviruses. These in turn can regain, by other mutations, the ability to act as viruses; that is, to become transmissible from cell to cell. Other elements of the bacterial genome may also become transmissible as a result of mutations.

In plant and animal viruses, we often find that the transmitted genetic material is ribonucleic acid (RNA). It has not yet been demonstrated that an RNA virus infecting a cell and persisting in it more or less permanently can cause any persistent changes in nuclear function, either structural changes in the chromosomal material or changes in gene functions. Yet an RNA virus may well cause the cell that it infects to acquire some persistent genetic characteristic. In general, changes in cytoplasmic or nuclear heredity may arise either from changes that occur within the cell or from the entrance into a cell of extraneous elements of genetic material (or from the loss of a fragment of genetic material that had come from outside).

Extraneous genetic elements that might cause changes in somatic cells need not be the usual "viruses." They may derive from a migration of genetic fragments, nuclear or cytoplasmic; for example, microsomes. RNA virsuses may actually be abnormal microsomal forms.

DANIELLI [2]: It seems to me that you are being very cautious, Dr. Ephrussi, on the inheritance of information about the formation of structures. We have obtained a great deal of information about this in the last few years by transferring nuclei back and forth between different strains of amoebae. Now the thing that has come to our notice very prominently is evidence of either nuclear or cytoplasmic control, or both, in relation to structure and function. The questions that then arise, however, are: What is the distinction between these two types of control? and What is their individual contribution as opposed to their joint contribution?

The distinction we have been considering is that maybe the nucleus carries the information about the formation of macromolecules involved (which should contribute to the structure), whereas the cytoplasm as far as we can judge seems to carry a very great deal of information about how these molecules can be put together into structures.

I do not maintain that these are the only two relations between nucleus and cytoplasm. There are, in fact, one or two alternative systems; but as far as I know, there is as yet no evidence for them. But I do think that we should distinctly separate in our minds the information that is necessary for the making of molecules from information that is necessary for putting them together into really specific and defined structures.

EPHRUSSI: I agree with you, and that is why I made the reservation in the concluding sentences of my paper. However, as I pointed out, I was not going to discuss here the problems of general cytoplasmic organization.

[2] J. F. Danielli, University of London Kings College.

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THE CYTOLOGY OF ANTIBODY FORMATION

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TWO FIGURES

Inclusion of a paper on antibody formation in a conference on somatic cell variation is appropriate, because antibody synthesis certainly represents a variation in cell function impressed by an external stimulus. Moreover, this alteration may persist for many years and hence raises the question of the method of its transmission through several or many generations of cells.

Burnet and Fenner in 1949 collected and discussed the evidence establishing the long persistence of specific antibody formation. They formulated the proposition that, once begun, synthesis can proceed in the absence of antigen and that synthetic ability must be transmitted from mother to daughter cell. They emphasized the importance of the difference in response between the first and subsequent exposures of the antigen to any understanding of the cellular mechanism by which antibody protein is formed. They also pointed out that the rise is logarithmic between the second and fourth days and suggested that this represented replication of some biological unit.

In a restatement, Burnet ('56) suggested that ribonucleic acid (RNA) templates are formed complementary to the antigenic determinants on the foreign molecules, probably in the cell nucleus. The RNA templates are then replicated, passed to the cytoplasm of another cell, and these, perhaps after further replication, serve as templates for the synthesis of specific antibody. This is a bald and very abbreviated summary of a

wide-ranging and thoughtful speculation. Moreover, it is a summary restricted narrowly to antibody synthesis, a response to an antigenic stimulus, and neglects other aspects of the theory dealing with the lack of antigenicity of autologous macromolecules, induced immune tolerance, and other important questions.

My colleagues and I (Leduc *et al.*, '55) have addressed ourselves to these questions by examining the cells engaged, or suspected of being engaged, in this process of antibody synthesis. The data drawn from these morphological studies will furnish the basis for my discussion. It is useful, I think, to divide the process of antibody formation into two distinct stages: first, the entrance of antigen into the cell and the gradual development there of a new ability, the ability to respond to the second injection. This new ability lies latent, however, until it is revealed by a second exposure to the same antigen or a closely related one. The second stage is synthesis of antibody protein, which occurs after repeated exposure to the antigen. It is carried out in cells that evidently spring from some sensitized precursors differentiating through several stages from a primitive cell to a mature plasma cell, multiplying a few times in the process, and synthesizing antibody on the way. Finally they disappear, a few mature cells containing antibody persisting for many months. Some of these persisting cells contain round hyalin objects known as Russell bodies that, as shown by White ('54) and Ortega and Mellors ('57), contain antibody or globulin.

The evidence for the important features of this summary is as follows:

After a first injection, antigen can be found in the cytoplasm of the cells lining the sinuses of the lymph node and also in cells lying between the sinuses. Although difficult to demonstrate, it has been seen on two occasions as small round dots inside the nuclear shadow of the reticular cells in the lymph node, sometimes in the nuclei of the cells with no demonstrable antigen in the cytoplasm. These reticular cells are undif-

ferentiated cells lining the sinuses and lying between them. Those between, at least in the spleen, are demonstrated in the electron photomicrographs of Weiss ('57). They form part of the wall of the closed sinuses; the sinuses evidently are alternately open or closed. Although these cells may differentiate into every active phagocytic macrophages, they need not do so.

Within a few hours after one injection of antigen, thousands of cells containing antigenic material are seen scattered throughout the medullary areas and around the follicles. Many of these cells are of the variety that we know could respond by making antibody. Occasionally there are also traces of antigen in the lymphoid follicles.

Of the thousands of cells that contain antigen 1 day after the first injection, only about 50 contain antibody on the fourth day. It is therefore not the usual response of a cell that picks up some antigen to synthesize antibody. Four days later, on the eighth day, there are still very few cells containing demonstrable antibody. They have become smaller and evidently contain more antibody because the cytoplasm is brighter; and they are perhaps slightly more numerous, but they do not double in number during the 4-day period.

The extent of the secondary response varies with the time between the two injections, but there are no systematic studies on this that I know of beyond that of Barr and Glenny ('45), in which they measured the peak titer in guinea pigs after injections of diphtheria toxoid 1, 2, and 3 months after the first injection. The peak was higher as the time was lengthened between injections.

On the second day of a secondary response, one can see many hundreds of cells containing traces of antibody scattered throughout the same areas where the antigen was deposited after its initial injection. These cells seem to spring up independently and do not form obvious colonies at the start. They do multiply, however, and their descendants often coalesce to form clumps of cells. But since these cells rise inde-

pendently in many areas, it is clear that they have not sprung from a single cell. They are not clones, even later when they look like clones.

Some improvement in our methods has enabled us to examine some of the finer features of these cells. Although there is diffuse antibody in the cytoplasm, there are often bright spots in the cytoplasm containing higher concentrations of antibody. Not only can spots be found in the cytoplasm, but also one can find local areas of antibody inside the nuclear shadow.

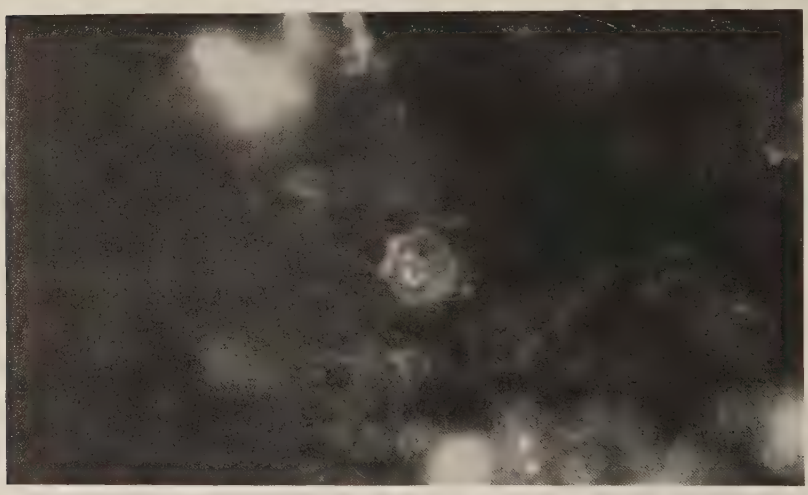

Fig. 1 Cell nucleus containing antibody.

Figure 1 shows a nucleus with antibody in the cytoplasm around the edge and a rather complicated structure within the nuclear shadow containing two or three bright granules of antibody.

We think that these objects seen in the nucleus are quite clearly nucleoli and that antibody formation either begins in or is intimately associated with the nucleolus of the sensitized cell. Lately, H. B. Donald and I have been examining such lymph nodes with the electron microscope as well as with fluorescent staining. Obviously, the sampling problem for

electron microscopy in the heterogeneous population of a lymph node is difficult. The striking find is the presence of very complicated nucleoli. We cannot be sure, of course, that these nucleoli are actually in cells that are synthesizing antibody, but the presence of such nucleoli is much more common in lymph nodes making a secondary response to an antigenic stimulus than in an unstimulated lymph node.

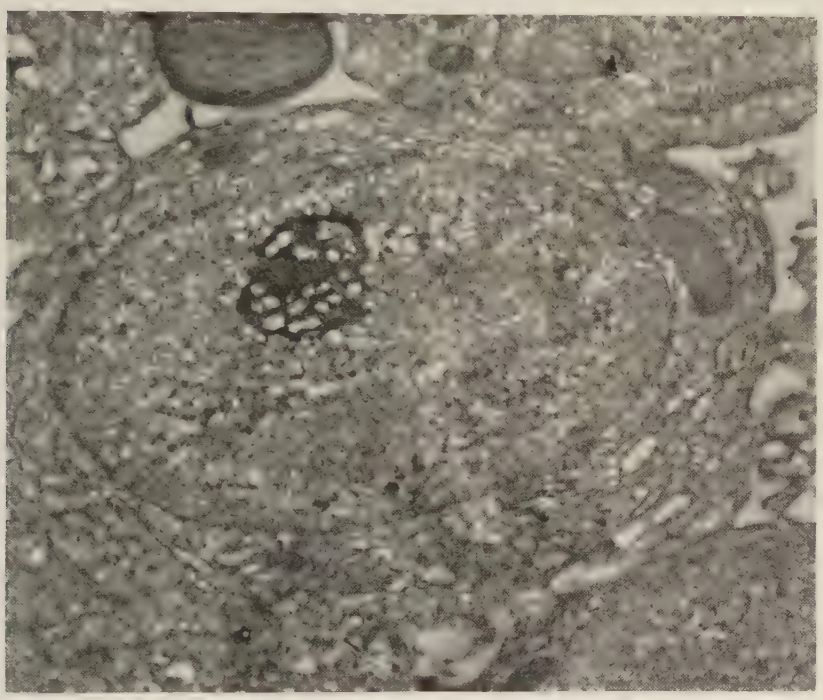

Fig. 2 Electron photomicrograph of a lymph node, showing a cell with a complex nucleolar shadow with several electron-transparent vacuoles in it.

Figure 2 shows a cell with a complex nucleolar shadow with several electron-transparent holes in it.

The cytological findings are that RNA increases in these antibody-forming cells. This is consistent with the current theory that RNA serves as a template for the specific synthesis of the antibody. Moreover, the finding of antibody in

intimate association with the nucleolus and the great prominence and complexity of that structure in the presumably active cells suggest the possibility that the site of template formation is located there.

Whether the alteration in the cells after sensitization is more deep seated than a modification of RNA is a proper question because of the long-lasting nature of the acquired ability to make a response to an injection of antigen. Although it is difficult to see how a protein molecule could proofread the poetry in the gene, I ask you to consider whether an additional bit of information could not be added to the end of a DNA helix?

OPEN DISCUSSION

Luria [1]: Is there any specific evidence that the less-scattering areas seen in the nucleoli are associated with antibody? Why would an accumulation of antibody give less-scattering region?

Coons: I am not quite sure I understand your question. The cells that I showed you — stained for antibody and inside the nucleus — contained a rather indefinite, slightly fluorescent structure, that I believe corresponds to the nucleoli visible under the light microscope and also in the electron microscope. Because of the geography of these objects in the electron microscope and in the fluorescent microscope, we suspect that the bright dots of antibody that we see under the fluorescent microscope correspond to the holes that we see in the electron microscope.

I am afraid I do not know the relative opacity of the RNA in the nucleolus versus the protein in these, what we might call, vesicles in the nucleolus. Evidently, the opacity is higher around the vesicles than it is inside them. For that matter, of course, the methods of fixation, and so on, are different. It may be that the antibody has been leached out of the nucleolus before we have had a chance to look at it under the electron microscope.

[1] S. E. Luria, University of Illinois.

UPTON [2]: I am quite excited by the observation that whereas with the initial injection of antigen the number of reticular cells that make antibody constitutes a very small percentage of the total that take up the antigen, on the second injection, if I understand you correctly, the number of antigen-containing cells that make antibody is relatively very large. From what you have observed about the relation between the time after the initial injection of antigen and the second injection of antigen, what are you able to formulate in the way of a mechanism that promotes a greater number of such cells to make the antibody on the second challenge? Is this a multiplication of such cells or is there some interaction between cells?

COONS: I think there are several possibilities, and I cannot solve the problem. I really was anxious to present it to an audience like this one. One possibility is that the initial change conferred by a first exposure to antigen has a very long latent period, so that only a few cells make any immediate response by synthesizing antibody, and, as time goes on, more and more of them become capable of it without any multiplication of anything — particles or cells.

The second possibility is that some of the cells become altered in a specific way, and during the normal course of their slow multiplication they increase in number. The alteration is transmitted from mother to daughter cells so that the number of altered cells gradually increases.

The third possibility is that there is a multiplication and spread of some kind of particle from a cell in which it is made to other cells, and that these particles can replicate and confer on the cell the ability to synthesize antibody when the second injection is carried out. That is about all I can say about it.

OWEN [3]: Dr. Coons' abstract has this sentence in it: "When a mixture of two antigens is injected, most of the cells engaged in antibody synthesis contain one antibody or the other,

[2] A. C. Upton, Oak Ridge National Laboratory.

[3] R. D. Owen, Oak Ridge National Laboratory.

none or few containing both." I do not think you mentioned this point in your discussion. Have you additional recent evidence on this, and what significance do you assign to it?

Coons: Thank you. I neglected to mention that, Dr. Owen.

Two or three years ago, Dr. N. Tanaka and I carried out a few preliminary experiments, injecting egg albumin and diphtheria toxoid into the footpad of a rabbit and injecting the same mixture of antigens a month later. We then had alternate sections stained for anti-egg albumin or anti-diphtheria toxoid, or both. When we did that we found that the response to egg albumin and to diphtheria toxoid was about equal, and that the two slides, stained for one or the other, contained about the same number of cells with antibody in them. But when we stained for both antibodies simultaneously, the number of visible cells that fluoresced was about double. We therefore thought that, in general, a cell makes one kind of antibody at a time. Such an experiment does not exclude the possibility that there may not be a few cells there that are making both antibodies.

I am told by my former colleague, Dr. White, that he has done similar experiments, with the same result. But there is much more-elegant work on the subject by Nossal and Lederberg. Perhaps Dr. Lederberg would be willing to say something about it.

Lederberg[4]: Dr. Coons is referring to a preliminary note by Dr. G. J. V. Nossal and me in *Nature* ('58). Cells from lymph nodes of rats hyperimmunized by footpad inoculations of a mixture of *Salmonella adelaide* and *S. typhi* were isolated in microdroplets and tested for their ability to produce anti-flagellar antibody against each of these serotypes. The antibody was detected by the immobilization of a few Salmonellas of each type introduced into the droplets some 4 hours after the lymph node cells were deposited. About 10% of the cells produced antibody against either serotype; no single cell was active against both. This conclusion must however be viewed

[4] Joshua Lederberg, University of Wisconsin.

rather cautiously. The method as used was not sensitive enough to detect a lower rate of formation of a second antibody; we have no direct assurance that each potential antibody-forming cell was equally stimulated by both antigens. In any event, we have no evidence that the apparent restriction to form a single antibody would be a *heritable* trait of the individual cells. The question of exclusion should also be tested with unrelated as well as related antigens. We are pleased to note, however, that these results are in accord with Dr. Coons' report.

I understand that Cohn and Lennox have been engaged in some experiments with a possibly more precise system: perhaps Dr. Lennox will comment on them.

LENNOX[5]: I would like to accept Dr. Ephrussi's precept and avoid confusion; therefore, I will await the results of experiments in progress to tell you whether we think one cell can make more than one antibody.

Dr. Melvin Cohn and I have been collaborating in experiments similar to those of Nossal and Lederberg. Using lymph node cells from rabbits immunized with two noncross-reacting bacteriophage, T2 and T5, we have succeeded in freeing them of background and in measuring antibody formed or released in microdrops. The fraction of cells inactivating either bacteriophage can be as high as 10%, depending on the course of immunization. We would like to await the results of cleaner experiments to feel confident that we know the answer to the question at issue here.

BURNET[6]: I merely want to make one remark. Dr. Coons gave a beautiful demonstration, but I would take issue with him that it has yet to be shown that the cells that take up the antigens are necessarily those that produce the antibody. I am rather impressed that you can show the taking up of antigen very well, indeed, in the liver; but there is no evidence, as far as I know, that the Kupffer cells ever make antibody.

[5] E. S. Lennox, University of Illinois.
[6] F. M. Burnet, Hall Research Institute, Melbourne.

As Dr. Lederberg hinted, we have been trying to develop in Melbourne a somewhat unorthodox point of view regarding antibody production. I do not want to say anything about that here, but I would mention the possibility that contact with antigen may act as a specific stimulus to proliferation for primarily conditioned cells. This may play a major part in determining the secondary response that Dr. Coons has illustrated so beautifully.

FRIEDBERG [7]: I would like to tell of some findings that are perhaps relevant to Dr. Burnet's comment that the cells that take up antigen are not necessarily those that produce antibody. In a study of the metabolism of radioiodine-labeled tobacco mosaic virus in mice over a 5-week period, I found a more rapid turnover in the spleen, an organ with antibody-producing potentiality, than in the liver, where little or no antibody can be produced.

SALK [8]: What I have to comment on bears on some experiments in which we were unable to demonstrate a secondary response in a particular way. I would be interested in knowing what explanation might be provided from your observations and of any hypothesis concerning the mechanism.

The experiment was done in the following way: The antigen used consisted of a suspension of crude tissue culture cell mixed with an additive. Groups of monkeys were given two doses, 2 weeks apart, tenfold increasing dilutions or diminishing concentrations of antigen additive. The first group received the undiluted suspension in two doses 2 weeks apart, and 4 weeks later received a second injection of the same concentration of material: complement-fixing antibody appeared promptly and began to decline. The second dose was given and there was no further rise, or a very slight rise, and then a prompt decline.

When, however, the next group of animals were prepared with the 10^{-1} dilution of antigen a lesser response occurred,

[7] Wallace Friedberg, Oak Ridge National Laboratory.

[8] Jonas E. Salk, University of Pittsburgh.

a decline. The booster dose this time was not 10^{-1} but the same concentration used in the first instance. The 10^{-2} dilution of antigen was used for preparing the animals. There was a very slight rise, a decline, and then a very sharp booster effect, which persisted in that way. A dilution of 10^{-3} produced no antibody rise in the primary stage and a very sharp rise here again. And 10^{-4} produced no primary response and only a faint secondary response.

The question that I raise is the explanation for the failure in one place and the response in the other. The second stimulus in all cases consisted of the same concentration of antigen, and so we have essentially a dose-response effect for the primary, and the effect of the secondary was quite the reverse.

The antigen consisted of a suspension of monkey heart cells grown in continuous culture suspended in distilled water and frozen at — 20°C. to rupture the cells. This same antigen was used in a complement-fixation reaction, and these antibody reactions were complement-fixing antibodies intramuscularly; the material was mixed with mineral oil adjuvant.

Coons: I would like to respond to Dr. Burnet's comments and then to Dr. Salk's comments.

Certainly antibody formation is the property of a specialized group of cells. There is no evidence that the Kupffer cells in the liver ever make any antibody from various kinds of experiments. They are known not to make γ-globulin. We have never been able to find any antibody in the Kupffer cells of hyperimmune animals, and the Kupffer cell evidently does not synthesize antibody. There are many other cells into which antigen penetrates that also do not make any antibody — hepatic cells, renal tubular cells, fibroblasts. Exposure to an antigen or to a potential antigen, then, is not all that is necessary to stimulate antibody formation.

It is true, as Dr. Burnet says, that we cannot find antibody and antigen in the same cell, but we can find antigen in cells in the same location, where subsequently we find antibody. There is a dark period during which we cannot find either. But I

personally do not have much hesitation in supposing that the antigenic stimulus reaches the cell directly and not through the mediation of other cells or by cellular teamwork, which I suppose you have in mind.

Dr. Salk's experiment is a little difficult to explain, but in the first place, the antigenic mixture of myocardium contains compounds of many different sorts, macromolecules of protein and polysaccharide, some of which might tend to persist so that a larger dose might be quite persistent and the apparent secondary challenge not a real one because the first antigenic dose is still present.

The second thing is that one can perhaps assume this even more strongly because the antigen was suspended in an adjuvant mixture. In at least some cases we know that mineral oil-antigen emulsions deposited in an animal contain demonstrable antigen for months afterward. So with a big initial dose one might suppose that the animal is already making all of the secondary response he can because he is being continuously stimulated by the slow release of antigen and the second dose really has no physiological effect.

SALK: Would you expect that animal would be refractive to antibody formation if another antigen was administered?

COONS: No. Is it?

SALK: I don't know.

RAPPAPORT [9]: In regard to the question of whether a single cell can produce antibody to more than one particular antigenic determinant, I think it is important to separate this problem from the additional complication of whether a single cell can take up two different kinds of particulates, whether they are bacteria or phages. The cases described for *Salmonella* and the T phages have added this unnecessary difficulty to the initial problem. I would suggest that, with the T phages, one has perhaps the best opportunity to decide whether or not a cell can make two different antibodies. Since the phages have a head and a tail antigen, there are three possibilities

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for antibody production. The cell may produce antibody that is directed against the head, against the tail, or against both. For this reason, screening should not be restricted to just the neutralizing antibody but to the agglutinating one as well.

The same criticism applies to *Salmonella*. Again, using a single type, one could screen the antibody-producing single cells for both antiflagellar and antisomatic antibodies.

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ERYTHROCYTE ANTIGEN MOSAICISM

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This review concerns animals with erythrocyte antigen mosaicism; i.e., a mixture of two or more populations of erythrocytes differing in one or more inherited antigenic properties. The words "erythrocyte" and "antigen" are both essential here in restricting me to my assigned topic; for there could doubtless be erythrocyte mosaicism with respect to characteristics other than those detected serologically, such as inherited hemoglobin types. But also, since some cellular antigens, including some blood group antigens, are not restricted to blood cells, there could be antigen mosaicism, even blood group mosaicism, without mixture of erythrocytes.

Consideration of the last two remarks may reveal two of the exceptional advantages that erythrocyte antigens promise for the study of somatic cell variation. Suppose we were to encounter a blood containing a mosaic of erythrocytes, 80% possessing the abnormal hemoglobin C and 20% lacking this substance. I am told that the chemical procedures used in this field would be quite incapable of showing whether C is distributed evenly throughout the red cells or, alternatively, confined to a portion of them. Perhaps this determination will one day prove possible, not necessarily by serological techniques. In erythrocyte antigens, however, the remarkable sensitivity of an agglutination or hemolysis test permits the identification of genetic differences in individual cells.

But imagine further that the gene for normal hemoglobin A mutated to that for C in some nonhematopoietic tissue, such as the epidermis, iris stroma, or parotid gland. Could the C gene be detected in such tissues? For some blood group anti-

gens, such as ABO in man, there is at least a strong hope that we may one day be able to investigate mutational changes in almost all tissues of the body — a prospect almost unique in genetics. The ABO substances have been shown to be present in extracts of almost all human organs (Hartmann, '41), and existing information seems to suggest that the responsible genes may be fully autonomous in many tissues. Before this hope can be realized, however, or even put to adequate test,

TABLE 1

Classification of erythrocyte mosaics

I. *Chimeras.* Mosaics arising from the compounding of cells derived from different zygotes, or possibly zygote plus unfertilized gamete, hence differing by virtue of the usual processes of gene segregation and recombination.

A. *Natural chimeras.*
1. *Twin chimeras.* In cattle, sheep, man, fowl; made possible through chorionic vascular anastomosis.
2. *Other conceivable types.* (As yet undemonstrated in blood.)
Polyspermy, with merocytes contributing to soma: bipaternity in birds, lower vertebrates (?)
Maternal → fetal chimeras.
Fetal → maternal chimeras.

B. *Artificial chimeras.* Arising through parabiosis or transplantation.
1. In prenatal life (embryo, fetus). Rats, fowl.
2. In postnatal life, after irradiation. Mice, rats, dogs, monkeys.

II. *Mutational (nonchimerical) mosaics.* Mosaics arising through somatic mutation in the broad sense: gene mutation, somatic segregation or crossing over, chromosomal aberrations, or variegation mechanisms of several kinds.

many new techniques must be devised and much more background information will be needed concerning modifying genes and quantitative variations in tissue antigens within and between individuals. Even in the erythrocytes themselves there are some serious technical difficulties to be overcome before we can learn to recognize mutational mosaics with confidence.

Table 1 gives a tentative classification of erythrocyte mosaics according to mode of origin. The listing of categories corresponds roughly to the chronology of discoveries in this

field. This outline also indicates the terminology adopted. The first blood mixtures discovered by Owen in cattle were called "mosaics" by him and subsequent workers, but their human counterparts have come to be called "chimeras." The original use of "chimera" in biology for graft-hybrids in plants (Winkler, '07) makes this term seem very appropriate for these two-in-one individuals, whether they arise naturally or through artificial transplantation. The word "mosaic" will be adopted as a generic term, applicable to erythrocyte mixtures of whatever origin. Such a cover-all term seems desirable since it may not always be easy to decide how some blood mixtures have come into being. This may happen in animals by virtue of the small number of blood-typing reagents available in some species. In man, added difficulties will be encountered from the common practice of blood transfusion and, more recently, of marrow transplantation.

NATURAL CHIMERAS

Chimerical twins

In the early nineteen-forties at the University of Wisconsin, blood typing in cattle had reached a stage of development unequalled in any other species. By immunizing cows against the red cells of other cows, antisera detecting some 20 or 30 specific erythrocyte antigens had been developed and the genetics of these differences had been investigated. R. D. Owen then noted that cattle twins were almost invariably identical throughout in their blood types, which was surprising since identical twins in this species were believed to be relatively rare. Weak hemolysis reactions were however frequently noted when the cells of twins were mixed with certain sera, and closer scrutiny showed that such instances were attributable to mosaicism, a mixture of two distinct kinds of cell. But whenever mosaicism was apparent for two or more antigens, as was frequently the case, a parceling-out process showed that only two cell populations were present. Genetically, it was shown that each twin transmitted genes cor-

responding to the phenotypes of one of the two red cell populations.

As to the explanation of the mosaicism, Owen ('45) stated: "These facts are consistent with the conclusion that an interchange of cells between bovine twin embryos occurs as a result of vascular anastomoses. . . These cells are apparently capable of becoming established in the hematopoietic tissues of their co-twin hosts and continuing to provide a source of blood cells distinct from those of the host, presumably throughout his life."

It is perhaps characteristic of important and surprising discoveries in science that their full importance is not easily foreseen and that, in retrospect, they sometimes seem not very surprising after all. Synchorial fusion had long been known in bovine twin placentas, and as early as 1911 Tandler and Keller observed that this anatomical feature was apparently essential to the appearance of freemartinism in those female twins that were paired with males. Lillie ('16) made this correlation the basis of his hormonal control theory of the freemartin. Moreover, one might have expected the physical opportunity for chimerism in cattle to be seized upon. Medawar ('54), in an introductory lecture at another symposium, summarizes this point as follows: "It is well known that embryos are tolerant of foreign, even 'xenoplastic' grafts, and much of experimental embryology owes its existence to this dispensation; it is less well known that foreign cells introduced into embryos may live on into adult life, though their host would certainly have rejected them if exposure had been delayed until shortly after birth."

In 1953 Stormont *et al.* found blood group mosaicism in a pair of sheep twins of unlike sex, and freemartinism was demonstrated in the ewe at autopsy. Earlier that same year Dunsford *et al.* ('53) announced the first case of a human chimera, Mrs. McK., whose twin brother had died in infancy. Since then, two more cases have been discovered in England. The more-essential facts concerning these cases are summar-

TABLE 2

Human twins with blood chimerism

REFERENCE	SEX	AGE	INHERITED ERYTHROCYTES *		GRAFTED ERYTHROCYTES *	SALIVARY ANTIGENS	CHILDREN
		yr					
Dunsford *et al.*, '53	♂	Died 3 mo	—		—	—	—
	♀	25	61% *OO kk* Jk^aJk^b		39% $A_1.Kk\ Jk^bJk^b$	H	3
Booth *et al.*, '57	♂	21	86% $A_1O\ MSMs\ R^1r\ Fy^bFy^b$		14% $OO\ MSMS\ R^2r\ Fy^aFy^b$	A H Le^a	None
	♀	21	99% $OO\ MSMS\ R^2r\ Fy^aFy^b$		1% $A_1O\ MSM.\ R^1r\ Fy^bFy^b$	H Le^a	None
Nicholas *et al.*, '57	♂	29	61% $A_1O\ NsNs\ R^2R^2$	Le(a— b+)	39% $OO\ MsNs\ R^2r$	Non-secretor	None
	♀	29	49% $OO\ MsNs\ R^2r$	Le(a+ b—)	51% $A_1O\ NsNs\ R^2R^2$	H Le^a	3

* Only those antigenic factors are indicated for which mosaicism was found, the genotypes being inferred in part from data on parents and sibs. All other serological reactions were identical within twin pairs, except Lewis (Le) types described by Nicholas *et al.*; for explanation see text.

ized in table 2; each case was brought to light through the discovery of a partial agglutination on routine ABO typing of the female twin.

Chorionic vascular anastomosis can also lead to chimerism in birds. Using immune guinea pig sera specific for certain erythrocyte antigens in chickens, Billingham *et al.* ('56) found that blood mosaicism is consistently present in twin chicks arising from double-yolked eggs.

Although these natural chimeras leave no reasonable doubt about their mode of origin, there are some puzzling questions yet unanswered concerning the detailed consequences of the process.

Does the chimerism extend to tissues other than the blood? In the human twins, nuclear chromatin studies of the neutrophilic granulocytes suggest the presence of both XX and XY cells in ratios similar to those found in the erythrocytes (Booth *et al.*, '57; Nicholas *et al.*, '57). Evidently then, the exchange involves the stem cells of both erythrocytes and granulocytes. Other than in the blood, however, no evidence of chimerism has emerged to date. Lillie ('17) noted that there was no apparent blending of pigmentation features in bovine freemartins and their male cotwins. The human twins appear to be unmixed with respect to their salivary ABO antigens (see table 2). If the latter turns out to be consistent, we will frequently be able to ascertain which of the two cell populations was inherited and which borrowed from the cotwin. This has been true in all three human cases studied, and in the female partner in Nicholas's case the diagnosis was confirmed by the finding of type M (*MM*) blood in two of her children (table 2).

Blood chimeras would likely be unknown today were it not for blood groups. Yet these unique twins have already repaid the debt not only in increased knowledge about tolerance phenomena, but also in crucial information about some of our blood types themselves. Stormont ('49) made one such discovery in a very simple and revealing experiment. He

placed J-negative cattle red cells into the sera of J-positive cows and, on recovering them, showed that they had picked up the J antigen. What made him suspect that J might not be an intrinsic property of the erythrocyte was the fact that twins showing mosaicism for other antigens were never mosaic for J and were sometimes different in J. The same kind of conversion was also demonstrated *in vivo*. Later, Sneath and Sneath ('55) found that the Lewis-*a* antigen in human blood is also primarily a property of the plasma, and the Lewis types of twins described by Nicholas *et al.* ('57) illustrate what Stormont had noted for J in some bovine twins (table 2).

Is Lillie's hormonal interference theory of the freemartin in any way challenged by the discovery of chimerism? And in what way is the sex situation different in man and cattle? Since vascular anastomosis in bovine dizygotic twins, as evidenced by red cell mosaicism, has been invariably accompanied by freemartinism in unlike-sex pairs, this blood test has found an important practical application (Stone *et al.*, '52). In the human chimerical twins, no female partner has shown obvious sexual abnormality; two of these women have produced children. Nor have abnormalities in the males been mentioned.

Another peculiarity of the human cases is revealed in table 2. In the two more recently described pairs, in which both partners were examined, the ratio of the two cell populations was distinctly different in the cotwins, the "grafted" population being in the minority in three of these persons. That the inequality may have resulted from a gradual selective overgrowth of the host's cells at the expense of the cotwin's cells is further suggested by a follow-up report (Dunsford and Stacey, '57) on Mrs. McK. When originally examined in 1953 she possessed about 40% A_1 cells derived from her deceased twin brother. In her serum there was a little soluble A antigen but no detectable anti-A. Since that time she has given birth to two group-O children. Despite the absence of A in these children and despite its presence in some of her "own"

red cells, a weak anti-A antibody was detected in her serum during both pregnancies, while, at the same time, the soluble A antigen was no longer demonstrable. Furthermore, the proportion of A_1 cells in her blood (30%) now seems to be significantly lower than in 1953. The authors suggest that these changes may reflect a "partial breakdown of acquired tolerance to the A antigen."

In dizygotic bovine twins, synchorial fusion occurs at about the 10-mm stage, followed by anastomosis of the chorionic vessels at about the 15-mm stage, according to Lillie ('17). It is not universal, but probably occurs in over 90% of the cases. In a sample of 275 unlike-sex pairs tested for freemartinism by blood typing, Humble ('52) found mosaicism in 258 or 93.5%. Multiple chimeras arising through multiple anastomoses have been observed in triplets, quadruplets (Owen, '46), and in quintuplets (Owen *et al.*, '46). In sheep the process is evidently less frequent, being estimated at 5% by Stormont *et al.* ('53). In man it is presumably quite rare. Considerably later occurrence of anastomosis and cellular exchange in human twins could perhaps explain the failure of freemartinism as well as the suggested manifestations of reduced tolerance.

How widespread is the occurrence of intertwin chimerism in other mammals? Is it mere coincidence that the three species in which it has already been found are primarily uniparous? Or have multiparous mammals evolved more effective safeguards against the compounding of individuals in this way? Even if chimeras should arise only rarely in our favorite experimental species (cats, dogs, rabbits, mice, rats), our failure to recognize such occurrences could sometimes lead to serious misinterpretations in experiments involving litter mates. Stormont ('54b) and I ('52) have both pointed out some examples of statistical biases in nature-nurture analyses and in estimations of gene frequencies brought about by failure to recognize that the great majority of cattle twins are two-in-one individuals.

The study of chimerism is certainly one good reason for urging a more thorough exploration of blood groups in many animals, and we would wish that there were many more laboratories like that created by Professor M. R. Irwin at Wisconsin. But in the absence of blood typing, the occurrence of chimerism in vertebrates can be investigated by skin grafting, a somewhat more laborious but perhaps more efficient method. Anderson *et al.* ('51), seeking to devise a simple grafting test to distinguish one-egg and two-egg cattle twins, were surprised to find that all dizygotic pairs studied, including many pairs of unlike sex, were nevertheless fully tolerant to skin grafts from their cotwins. We can be thankful these well-designed experiments failed to achieve their intended purpose, for they led instead to further extensive studies on "actively acquired tolerance" in mammals and birds (Billingham *et al.*, '53, '56), some of the principal conclusions of which are alluded to in this paper. This work has gone a long way in affirming for tissue isoantigens a proposition first enunciated by Burnet (see Burnet and Fenner, '49; Medawar, '54): "if a young animal is confronted with an antigen before it is capable of responding by the formation of specific antibodies, then its capacity to do so in later life is reduced or wholly suppressed."

Other possible modes of chimerism

Hollander ('49) reported several cases of pigment mosaicism, in pigeons, that seemed to defy explanation on the basis of multiple mutations but could be readily explained by assuming that one or more supernumerary sperms from the same male or from different males had given rise, through mitotic proliferation as merocytes, to the abnormally pigmented areas. If this explanation proves well founded, we may perhaps look forward to the discovery of erythrocyte mosaicism from this cause in birds and in lower vertebrates in which merocytes have been observed. Such mosaic bloods would be expected to mimic bloods of chimerical twins in frequently revealing

mosaicism for several antigenic factors. But instead of just two cell populations there might be several, and maternal contributions would be demonstrable in only one.

Along with this, I might mention the possibility of chimerism between mother and fetus (embryo) in mammals, through transplantation from mother to fetus, fetus to mother, or both. No cases of blood mosaicism yet found in man or other animals suggest these happenings, and blood typing can of course easily dispose of such hypotheses. Billingham *et al.* ('53) remark that they have inquired "into the possibility that it [actively acquired tolerance] may occur naturally by the accidental incorporation of maternal cells into a foetus during normal development." They cite ('56) the fact that malignant melanomas have been known to pass the placental barrier and establish themselves in the tissues of the child (see also review by Wells, '40).

The reverse kind of implantation would seem much less likely to succeed, although the placenta itself is a kind of natural homograft and some pregnant animals exhibit a somewhat prolonged acceptance of skin homografts (Medawar, '54). And again, chorionepithelioma might be cited as an example of pathologic invasion of fetal cells into maternal organs (Boyd, '43). In this connection, it may be of interest that Reich ('32) found that the human decidua possesses A antigen in group-A mothers and the amnion possesses A antigen in group A fetuses, but the chorion appears to be a kind of neutral zone, lacking group specificity.

ARTIFICIAL CHIMERAS

Blood groups have also served to verify the creation of artificial chimeras arising from transplantation or parabiosis in embryos. Weiss and Andres ('52) injected suspensions of dissociated cells of chick embryos into the embryonic circulations of other chickens and ducks belonging to white breeds. Chimerism was demonstrated by the persistence of colored melanophores characteristic of the donor breeds. Since whole

embryos and parts of embryos were used in preparing the inocula, blood chimerism may well have been present, but this was not tested. S. Ripley and R. D. Owen ('53, unpublished observations) observed persistent erythrocyte mosaicism in rats after injection of embryonic cells into rat embryos of different blood types. Billingham *et al.* ('56) produced red cell chimeras in chickens by a modification of Hašek's parabiosis technique, forming chorioallantoic bridges between 10-day-old embryos. Rabbit antichicken hemolysins were used for the blood typing. Although red cell chimerism persisted for months in some parabionts, it was not permanent; survival of exchanged skin grafts in these same pairs paralleled persistence of erythrocyte mosaicism.

In the transplantation studies on embryos, chimerism was the deliberate objective. In similar experiments on irradiated adult animals, its discovery came as a dramatic climax to 4 years of intensive research in several laboratories (review by Congdon, '57). Lorenz *et al.* ('51) showed that adult mice and guinea pigs could be protected against the lethal action of radiation by injection of isologous bone marrow into the irradiated animals. Jacobson ('52) discussed the possibility that the depleted marrow might colonize, but gave primary consideration at first to humoral factors thought to be assisting in the recovery of the host's own hematopoietic tissues. Then, in rapid succession, several proofs of chimerism were brought forth. Lindsley *et al.* ('55) used immune anti-C and anti-D rat hemagglutinins to demonstrate the persistence of large numbers of implanted red cell precursors in rat-to-rat transplants. Heteroimmune red cell agglutinins were used by Makinodan ('56) and by Vos *et al.* ('56) to show that replacement of mouse erythrocytes by rat red cells occurred after injections of rat marrow. Also, heteroimmune platelet agglutinins served to demonstrate that rat platelet precursors are successfully implanted in the rat-to-mouse experiments (Smith *et al.*, '57). Histochemical and cytological observations have led to similar conclusions for granulocytes. Sev-

eral reviews and contributions in this field were published in the 1957 symposium of the Biology Division of the Oak Ridge National Laboratory Symposium on Antibodies: Their Production and Mechanism of Action (J. Cell. and Comp. Physiol., *50,* Suppl. 1).

The relation between blood groups and transplantation studies can be one of mutual assistance. Cellular antigens will probably continue to provide the most convenient means of marking transplanted cells. In turn, the broadened knowledge of immunity and tolerance derived from these experiments will furnish an important guide for extension of blood types into another research field. This concerns a subject as yet little explored: somatic variation and mutation in blood group antigens.

MUTATIONAL MOSAICS

Of the two general ways in which genetic mosaicism can arise, through chimerism or somatic mutation, we would guess that mutation is by far the more common in plants and animals. Here the term "somatic mutation" is used in the broad sense, referring to any process of genetic change occurring within the somatic tissues (table 1). If this should eventually be found to hold for blood-cell antigens, we might then ask why mutational erythrocyte mosaics have not been found rather frequently in man and cattle, the two species most intensively studied.

Doubtless one reason may be that too little thought has been given to the possibility that they do occur. On the other hand, mutational blood mosaics can be expected to be more difficult to discover than chimeras. The latter will generally present three distinguishing features: (1) the twinning itself, (2) the presence of mosaicism for two or more antigens simultaneously, and (3) a not very unequal mixture of two cell populations. None of these, of course, is an infallible criterion for chimerism. We have noted that natural chimeras may sometimes have decidely unequal mixtures. If the twins are unusually similar antigenically, or if only a few blood-typing

reagents are available, the mosaicism may be noted in only one system or, of course, none at all. And even twinning may not be in evidence. Stormont ('54a) recorded an instance of mosaicism for four antigenic factors at two gene loci in a calf recorded as single-born; presumably, the cotwin aborted or resorbed after chimerism had been established in the usual way. Conversely, to make matters more confusing, blood chimerism may be observed in monozygotic twins! This can happen, for example, as a consequence of a three-way anastomosis in two-egg triplets. A case of this sort in cattle was reported by Gilmore ('52, personal communication).

Simultaneous mosaicism for two or more blood groups clearly increases the chances for discovery of a chimera. But, more important than this, each antigenic difference found in the cells of a chimera serves to vouchsafe the uniqueness of the partition effected by each of the other sera. Some mutational events, such as deletion, somatic reduction, and crossing over, could of course bring about changes in two or more antigens simultaneously. But until we have many more antigenic markers to work with, we will usually be confronted with a single difference. This means that it will be very difficult to demonstrate a distinct dichotomy underlying the observed partial agglutination or hemolysis reaction. If the two phenotypes agree in all respects with those known to be genetically controlled in whole individuals, this will help greatly. Yet this criterion could fail if certain kinds of mutation responsible for unusual red cell phenotypes were lethal when they occur in the germ line.

Much of the literature on mosaicism in plants and animals deals with pigmentary differences, and here the fixed nature, size, and shape of the spots frequently helps to convince us that mutation occurs. In some cases, as in "twin spots," we may even gain important clues about the nature of the mutational event. The fact that blood is a constantly mixing tissue is also a decided handicap in another way: if one-one hundred thousandth of my body surface possessed an abnormal pig-

ment genotype, this might make an easily visible spot measuring 20 mm^2. But if my blood were mosaic for the Kell antigen, say, with only one cell per 100,000 possessing Kell antigen, the detection would be wholly impossible by present techniques. Yet there is one consoling feature: a single drop from any finger or vein would contain about 2500 aberrant cells to be looked for.

Even though mutations occurring in the erythropoietic tissues might accumulate throughout life, if the mutations were rare and were selectively neutral or subneutral, most mutational mosaics would be of the minute variety, i.e., presenting only a very small admixture of cells of the mutant types. Before considering these cases, we may note some exceptional conditions under which we might anticipate more nearly equal mixtures arising through mutation. These include (1) gene instability or variegation; (2) neoplastic blood diseases; and (3) very early gene mutation or chromosomal aberration, the latter probably often associated with other visible forms of mosaicism. As possible illustrations, I shall briefly summarize three reports of unusual findings in human blood type in which somatic mutational changes have been postulated. In these cases an irregularity was found in the ABO groups but in no other blood type system tested. No individual had a history of twinning or of transfusion.

$O + A_2$. Cotterman ('55) reported two families with $O + A_2$ mosaics; $A_2 = 8$–12% in four cases. The first case was a woman who had been a donor on six occasions, three times being pronounced O and three times A. A clear-cut partial agglutination (12% in one or a few large clumps) was observed in 87 O and 18 B sera, including those with low anti-A titers of 16 or 32; the same was true in very potent anti-A reagents of human, animal, and plant origins. A variety of tests failed to show that the O cells absorb anti-A. The picture has remained unchanged during 3 years. The woman has a marked heterochromia iridum partialis. The A antigen was

found in one small area of the decidua parietalis after the birth of a third group-O child. The mosaicism is evidently inherited, since it was present in a younger sister. In a second family (Di) a similar $O + A_2$ mosaic was found in mother and son.

$A_1 + O$. Salmon *et al.* ('58) describe a 32-year-old man with acute myelogenous leukemia, who, for 76 days before death, exhibited an $A_1 + O$ mosaic; $O = 25\%$. His mother and brother are O; his father, wife, and one child are A_1, without obvious mosaicism. The authors believe that somatic mutation, deletion, or nondisjunction are the most likely explanations. No earlier blood typing had been performed, so there is no evidence bearing on the question whether the mutation might have been induced by radiotherapy over the spleen (11 treatments) or by antimitotic drugs (urethane, myleran) administered during the 2 previous years of illness.

In another report suggesting some form of somatic change, van Loghem *et al.* ('57) describe a leukemic patient who was once typed without difficulty as group A in two laboratories, but a year later was found to have red cells slightly or not at all agglutinable with anti-A sera, though strongly absorbing anti-A antibodies.

O Erythrocytes, B saliva. A number of secretors have been described in which there is a discrepancy between the blood group as tested on the saliva and that determined on the red cells. In such instances, recessive modifying genes have sometimes been offered as explanations. But in another case, Armstrong *et al.* ('57) thought such an explanation needed modification since the person having O erythrocytes but a saliva containing B substance in nearly normal quantity was a bilateral gynandromorph or true hermaphrodite; (testis on right, ovary on left). The authors postulate a recessive gene inhibiting B in the erythrocytes, which, through translocation between the X chromosome and an autosome carrying the inhibitor's dominant allele, left the red cells without B antigen.

No mosaicism of the erythrocytes was evident for any blood type factor. The typing indicated the following genotype:

$$OO,\ MsMs,\ P_{1},\ R^{1}R^{1},\ Lu^{b}Lu^{b},\ Kk,\ \mathrm{Le(a-b+)},\ Fy^{a}Fy^{a},\ Jk^{b}Jk^{b}.$$

This seems rather remarkably homozygous; were it not for *Kk*, we might suppose the erythropoietic component of this individual to be derived from a haploid or amphihaploid cell.

In any partial or differential agglutination or hemolysis reaction, one portion of cell reacts positively and one negatively. Let p stand for the proportion of positive (agglutinated or lysed) cells. Under favorable circumstances, there will usually be little difficulty in verifying the existence of a mixture and in estimating p in the range $0.05 < p < 0.95$, or perhaps even in the range $0.01 < p < 0.99$. But at the extremes $p \rightarrow 0$ or $p \rightarrow 1$, we encounter increasing difficulty in deciding whether such residual cells are genuinely different from the remainder in their antigenic make-up. The technical difficulties, at least for agglutination, are quite different at the two extremes, and it is useful to distinguish these cases by the terms "minute positive" and "minute negative" mosaics.

If we assume that the mutant cells always constitute the minority class in any minute mosaic, the terms "positive" and "negative" also imply something about the kind of phenotypic change, although not about the nature of the mutational event. For example, an M + MN mosaic (where M and MN are phenotype designations, MN constituting the minority class) represents the minute positive, since anti-N will react with a small proportion of cells only. This implies, if MN really is the mutant class, that the change involved the gain of a new or foreign antigenic property N. But this might be attributable either to mutation at the *M–N* locus ($MM \rightarrow MN$), or to mutation or deletion of a dominant inhibitor of N at another locus ($MN, Ii \rightarrow MN, ii$ or $MN, Ii \rightarrow MN, -i$), or to yet other possibilities. Under some circumstances, origin of a new antigen by mutation might initiate an immune reaction, thus exposing the mutant cells to a mechanism of selection to which

the negative class (e.g., MN + M) would not be expected to be liable.

This classification will not always be entirely serviceable. For example, "positive" and "negative" are not mutually exclusive in this usage; in a mosaic of the sort $A_1O + BO$ (which could certainly arise through chimerism if not through mutation), the B cells could be detected both positively, with anti-B, and negatively, with anti-A. Moreover, a mosaic might comprise more than two red cell classes for any blood group system, e.g., MN + M + N, the last two classes arising through independent mutations or, simultaneously, through somatic crossing over.

Minute negative mosaics

Though it is convenient to speak of complete agglutination when referring to strong blood-typing reactions, it is perhaps seldom if ever true that hemagglutination is complete. Usually there is a small residue of free or unagglutinated cells even when very potent reagents are used under conditions thought to be optimal for agglutination. This so-called free-cell phenomenon has received relatively little study except in relation to a problem of practical concern. When red cells of a group-O donor are transfused into an A recipient and their survival measured at intervals by estimating the proportion of A-negative cells, a correction must be applied for the free-cell count of the recipient.

From a study designed to elucidate the nature of free cells in relation to antigens A, B, M, N, and Rh, McKerns and Denstedt ('50) concluded that such cells were not devoid of antigen and were, in fact, agglutinable by these same sera when carried over into freshly treated cells of the same individual. K. C. Atwood (this symposium) discusses this problem in detail. He has obtained evidence that at least some of the free cells found in *AB* and *AO* bloods are genuinely different in their serological properties and that these may well qualify as examples of the theoretical class: minute negative

mosaics. I shall therefore turn to the opposite end of the scale and ask you to consider an equally interesting category.

Minute positive mosaics

An example of minute positive mosaics would be a mixture of Rh-negative and Rh-positive cells of the sort $rr + R^1r$, the two cell types occurring in the ratio 999 rr:1 R^1r. Though mosaics of this sort are unknown, a deliberate search for them would seem promising, for the following reasons: (1) Such mosaics, especially when arising through early somatic mutation, might be expected to persist into adult life. (2) They would almost certainly escape detection in ordinary blood typing, the blood passing as Rh negative. (3) Certain peculiarities of the blood and of the person's immunological history might provide clues to his or her mosaic character in Rh. Thus, with more-sensitive methods of detection, a selective search for such cases might be undertaken.

Factors that favor persistence. It has already been noted that a new antigen, such as Rh^1, could make its appearance through loss of a chromosome or chromosome fragment carrying a dominant inhibitor gene that suppresses the development of Rh^1. Such instances of nondisjunction or deletion might well prove lethal through alterations of cell physiology in no way related to the antigen under consideration. Point mutation at the *Rh* locus, however, would not seem to involve anything beyond immunological effects of the antigenic change itself on the mutant cell and its descendants.

If the gene mutation ($r \rightarrow R^1$) occurred first in embryonic life, or perhaps in early fetal life, the descendants of the mutant R^1r cell might be expected to be tolerated in adult life just as those derived from a chimerical cotwin or transplantation donor. At least the challenging antigen would be present in living cells that differ from those of the host in this one genetic factor only. In only two respects would the situation here seem different: the foreign antigenic stimulus would be quantitatively smaller, both in the proportional

frequency of cells introduced (one per 1000 or less initially) and in the number of antigenic differences (here just one). There seems to be no experimental evidence to suggest that these smaller dose effects would lessen the chances for actively acquired tolerance. The prenatal mutations that confer tolerance would not have to occur in the erythropoietic tissues; other expendable and phagocytized mutant cells might be expected to have the same power to induce tolerance (Billingham *et al.,* '56). Sooner or later, of course, mutations would have to occur in the erythropoietic cells if red cell mosaicism were to result.

A maternal genotype R^1r is another possible factor that might favor the continued proliferation of the mutant R^1r cells, whether these arose first in prenatal life or later. Owen *et al.* ('54) found some evidence that anti-Rh antibodies are less likely to appear in Rh-negative women if their mothers are Rh positive, suggesting therefore that some measure of tolerance can be conferred by contact with the Rh antigen in intrauterine life.

In table 3, some possible consequences of recurrent antigenic mutations of the positive type are pictured, M and N being used as gene symbols instead of r and R^1. The conditions assumed to be most favorable to a persistent mosaicism are maternal antigen contact (case 1c), early occurrence of first mutation (1b), or both (1a). But it is not necessary to suppose that these are the sole factors, or even principal ones, governing sensitivity to an antigen. Especially, perhaps, for poor antigens, like N in man, one might envision the persistence of mutant MN cells despite the absence of the two mitigating prenatal factors (case 2). When tolerance is not fully established, an autoimmune reaction might result, and in this way irregular isoantibodies could appear without apparent antigenic stimulation. I have studied a man having the very rare anti-N as a natural antibody; as yet there is no evidence of mosaicism to support the idea of stimulation through somatic mutation. The antibody is of the cold type, and when

TABLE 3

Some conceivable consequences of gene mutation occurring in the erythropoietic tissues of a homozygote (MM) and producing a foreign or newly acquired antigen (N)

CASE NO.	MOTHER'S GENOTYPE	ZYGOTE	MUTATIONS OCCURRING IN ERYTHROPOIETIC CELLS OF: Embryo	Fetus	Adult	CONSEQUENCES
1a	*MN*	[*MM*]	*MN*	*MN*	*MN*	*MM* + *MN* mosaic; no anti-*N*.
1b	*MM*	[*MM*]	*MN*	*MN*	*MN*	Immune response negated or reduced in probability owing to maternal antigen *N* contact (1c), early mutations (1b), or both (1a)
1c	*MN*	[*MM*]			*MN* . . . *MN* . .	
2	*MM*	[*MM*]			*MN* . . . *MN* . .	*MM* + *MN* mosaic; no anti-*N*. Immune response not elicited; *N* is a poor antigen
3	*MM*	[*MM*]			*MN* * *MN* . . . *MN* . .	*MM* + *MN* mosaic; anti-*N*. Anti-*N* produced, but *MN* RBC proliferation not abolished
4	*MM*	[*MM*]			*MN*. . *MN* .	No RBC mosaic; anti-*N*. Anti-*N* produced, and *MN* RBC proliferation abolished
5	*MM*	[*MM*]			*MN* * * *MN* *	No RBC mosaic; anti-*N*. Anti-*N* stimulated by *MN* mutants in other tissues *

In this table, *M* and *N* could stand for any two allelic antigenic factors, but the human *M-N* blood types will also serve as a concrete example.

we recall the findings on Mrs. McK. (Dunsford and Stacey, '57), we might suppose that mosaicism could persist in the peripheral blood despite the occurrence of an immune response (case 3). With techniques now available, a mosaic comprising less than 1% *MN* could not be detected. But mosaicism would not be expected if the autoimmune response were to spell the extinction of the mutant stem cells in the marrow (case 4), or if the source of stimulation consisted of mutant *MN* cells in nonhematopoietic tissues (case 5).

Difficulties of detection. In minute negative mosaics, there may or may not be uncertainties about the interpretation of the unagglutinated cells, but at least these are free and present no special difficulty in the way of microscopic observation and of recovery. In minute positive mosaics, on the other hand, a free-cell phenomenon of a more troublesome kind is encountered.

To illustrate this, agglutination tests were carried out on artificial test tube mixtures of papainized, saline-suspended cells of genotypes *rr* and R^1r. A 2% suspension of R^1r cells was diluted progressively with a 2% suspension of *rr* cells. The cell suspensions were mixed with anti-Rh serum and put into the 10-mm test tubes, which were then mounted in a nearly horizontal position over the stage of a wide-field microscope to permit continuous observation. The time of first appearance of microscopic agglutination was recorded. The results, typical of experiments of this sort, are shown in table 4.

TABLE 4

Time of first observation of microscopic agglutination in artificial mixtures of papainized R^1r and rr erythrocytes

Reagent: Anti-Rh_0, titer 2048 versus R^1r
Reading: × 18 stereoscopic microscope

Proportion R^1r cells:	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Agglutination: time (min)	1.4	2.4	2.6	5.5	10.0	15.3	18.3	30.7	38.5	35.0	—

Not only are these agglutinates very tiny and hard to find in a large sea of unagglutinated cells, but also their speed of formation is markedly reduced as the proportion of positive cells decreases. This is interpreted as the partial result of physical interference by negative cells, which slow the rate of contact among the few antibody-coated positive cells. Centrifugation does not seem to help at the weaker concentrations, nor would this be expected, since the positive cells in the centrifuged pack are still separated by negative cells.

In routine testing, a mixture of 99 $rr + 1\ R^1r$ might easily escape detection even after prolonged observation, and particularly so if a more viscous suspension of cells in serum or albumin were used instead of the saline suspension of papainized cells used in the experiment cited in table 4. Attempts to demonstrate minute positive admixtures of the sorts O + A, O + B, $rr + R^1r$ have uniformly failed in my experience when $p < 0.002$ or $p < 0.001$. But doubtless more-sensitive techniques could be developed that would not require the agglutination *inter se* of the positive cells. Such methods are critically needed for the study of mutational changes in erythrocytes.

Those who are familiar with some of the intricacies of human blood groups have probably noted that I have assumed here that an R^1 (*CDe*) gene could arise from *r* (*cde*) by a single mutational change. This is a heretical thought if the concept of linked gene loci is favored. I could have avoided the issue by choosing a different example, say *r* (*cde*) $\rightarrow R^0$ (*cDe*). But it is clear that, if refined techiques should reveal somatic mutations in the Rh system, this could help to resolve the much-debated question of multiple alleles versus closely linked genes. Techniques by which very small proportions of positive cells could be detected would also be useful in a search for circulating fetal cells in the maternal blood stream. These cells might provide the basis for immunization in pregnancy.

Immunological peculiarities. A mosaic blood, $rr + R^1r$, containing only a small proportion of R^1r cells, might be ex-

pected to have some properties at variance with its classification as Rh negative. If the blood were of type B and were used to absorb anti-B from the serum of a group A patient possessing both anti-B and anti-Rh_0, some slight reduction in the titer of the Rh antibody might be observed. But if used in transfusion or for immunization (say anti-B) of animals, an anti-Rh antibody might unexpectedly appear in the immune serum. Nonspecific absorption is not uncommon in serum production work, and a few instances of nonspecific immunizations wherein antibodies have been produced against antigens apparently missing in the donor's cells have been reported. If mosaicism were the explanation, the paradoxical nonspecificity would be fully explained in both instances.

A more-interesting consequence of the mosaicism might be this: being tolerant of their own mutant cells, the mosaic persons might be expected to be partially or wholly insensitive to the Rh antigen in transfusion or in pregnancy. This suggests that we could make a selective search for Rh mosaics of the kind here considered, choosing Rh-negative women having large numbers of Rh-positive children without the production of anti-Rh antibodies. The same kind of search could be extended to patients receiving multiple transfusions, though this would not be feasible for antigens A, B, and Rh_0, since here cross-matching of donor and patient are now almost universal precautions.

Thomsen ('28) called attention to the fact that the isoagglutinins anti-A (α) and anti-B (β), which are normally developed after birth in infants lacking the corresponding antigens A and B, could sometimes be unexpectedly missing in adults. He found four such "defektive Blutgruppen" in a sample of 3500 bloods. These cases were the following: Oα(β), O(α)β, B(α), and Oα(β); the missing antibodies being shown in parentheses. If early somatic mutations could have forestalled the appearance of these missing antibodies, we would anticipate finding mosaicism of the sorts: O + B, O + A, B + AB, O + B. If the mosaicism involved the red cells it would

probably have escaped detection unless $p > 0.01$. But the responsible mutations need not have occurred in the hematopoietic tissues; the mosaicism might be sought in other tissues. It should not be inferred that somatic mutation is the only explanation of defective blood groups; more-thoroughly studied cases in recent literature suggest that modifying genes may be commonly operative. The problem is mentioned here merely to show another advantage afforded by the ABO groups in the study of somatic mosaicism. Red cells of the propositus in the $O + A_2$ family Di (Cotterman, '55) were at first classed as O in routine typing, until absence of anti-A (though not of anti-A_1 and anti-B) drew attention to a small proportion (8%) of A_2 cells. The cases described by Booth *et al.* ('57) and Armstrong *et al.* ('57) could also have been discovered in this way had there not been other indications (twinning, hermaphroditism) calling for a thorough blood type study.

If somatic mutation seems inevitable in large multicellular animals, we must seek to reconcile this with the principles of acquired immunological tolerance and acquired immunity. The word "acquired" in medicine and biology generally suggests external influences. But organisms also acquire genes, and some birds and mammals almost certainly acquire them in three ways: by inheritance, by somatic (and germinal) mutation, and by transplantation (chimerism) from a twin. The first and third modes of acquisition are now well recognized for their high effectiveness in ensuring immunological tolerance. Both are very early acquisitions and are noncumulative. Mutations, on the other hand, presumably arise at all stages of development; and, in the hematopoietic tissues, mutant cells can be expected to accumulate in the absence of negative selection. If the mutation occurred at the earliest opportunity, in the zygote, both the genetic and the immunological consequences might be expected to be identical with inheritance. Occurring later, the mutations, if not neutral, might be expected to lead either to specific tolerances or specific autoimmune reactions, depending largely, perhaps, on

the time and site of first occurrence. In this way somatic mutation might prove to be an important adjunct as well as an antagonist of heredity in shaping the antibody-forming capacities of an animal.

SUMMARY

It is now many years since F. Bernstein and L. H. Snyder first drew attention to the unique value of blood groups as genetic markers. They referred to linkage studies, wherein blood group genes serve to identify events in gametogenesis responsible for differences *between* individuals. After a rather discouragingly slow start, progress is being seen in this field. Now we are beginning to consider ways in which blood groups could be used as markers of genetic events responsible for differences *within* individuals. Here again the prospects seem very good, but the new line of work will demand special techniques and a much more thorough knowledge about antigens, antibodies, and their reactions. Some mistakes will doubtless be made. We are mere embryos in a new, fertile field. As embryos, of course, we can be excused for being vigorous or even exuberant, but we must also be patient and, above all, tolerant.

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SOMATIC VARIATION IN HUMAN ERYTHROCYTE ANTIGENS

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THREE FIGURES

The certainty that genetic heterogeneity exists in any large cell population has led us to an examination of human blood for exceptional erythrocytes that may result from somatic mutation in erythropoietic stem cells. Cells lacking A agglutinogen in normal A or AB persons were found and characterized. They comprise about one per 1000 of the red cells in heterozygous young adults, and their phenotypes agree in every way with the expected consequences of loss of the A allele. In a single preliminary experiment, some cells apparently lacking B agglutinogen were also found in AB blood, and these were phenotypically A. Although these experiments were prompted by the hope of revealing mutation rates and patterns, evidence so far gathered cannot settle the origin of exceptional cells (EC). General considerations can be cited, however, to indicate that at least some fraction of the exceptional cells originates through mutation in the broadest sense of the term.

Spontaneous mutability may be regarded as a general property of genes; cases of apparent immutability can be attributed either to deletion of the gene, when its total reconstitution would be extremely improbable, or to the stability conferred by a redundant or manifold structure. With respect to the normally mutable loci, it follows that cell populations of

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greater size than the reciprocal of the mutation rates will be genetically heterogeneous. Rates exceeding 10^{-9} per cell division are not unusual, hence the cell populations of most metazoan tissues are large enough to ensure heterogeneity. Although the presence of mutant cells is inevitable, nothing is known of the frequency of parallel phenotypic alterations of nongenetic origin. It is therefore a matter of conjecture whether, at a given locus, mutants in the erythropoietic stem line will be directly reflected in the composition of mature erythrocyte populations or outnumbered by phenocopies. Since the criteria of heritability and recombinatorial analysis cannot be applied, distinction between mutant and phenocopy will have to be based on other evidence. For example, one may study the distribution of exceptional cells among different individuals, the relation to the age of the individual, to heterozygosity, and to mutagenic agents. The altered phenotype can be described in detail and compared with those produced by known alleles of the locus. It will be seen that the mutational hypothesis leads to a restricted, if not unique, set of expectations by these criteria.

Loss of the A reactivity in AB individuals seemed a favorable starting point for several reasons. Transfused B or O cells are known to survive normally in AB blood, owing to the absence of isoagglutinins; hence such cells originating within the individual should complete their normal life span. Obvious chimerism for A agglutinogen is known, hence the A phenotype of the cell must be autonomously determined — another necessary condition for the detection of somatic mutants. The composition of mosaics such as those Cotterman described (this symposium) appears to be fairly stable, suggesting that compatible differences at the ABO locus do not of themselves cause selection pressures in the bone marrow. Finally, the easy availability of anti-A lectin from lima beans — discovered by W. C. Boyd in 1945 (see Boyd and Reguera, '49) — solved our major technical problem: the need for very large quantities of high-titer agglutinin.

ISOLATION OF EXCEPTIONAL CELLS

The preliminary experiments of April 1957 were done with AB cells and commercial anti-A and anti-B typing sera. Washed cells from 5 ml of blood were placed in a staining dish with 5 ml of saline and gently agitated while antiserum was added, 1 ml at a time. Visible clumping occurred after addition of 5–8 ml of antiserum. The mixture was then divided among eight tubes and lightly centrifuged so that only the larger clumps were brought down and the supernatant still contained free cells and small clumps. The first pellets were discarded and the supernatants recentrifuged. After withdrawal of all but a few drops of the resultant clear supernatant, the second pellets were thoroughly fragmented, combined into two tubes, and lightly centrifuged. Again the pellets were discarded and the free cells and small clumps in the supernatants concentrated. After fragmentation of the resultant pellets and resuspension in undiluted antiserum, the process was repeated until an inagglutinable residue was obtained. In the last stages, the supernatants containing free cells were withdrawn with a capillary pipette while under observation through a dissecting microscope. Six to seven stages were required to purify free cells with anti-A, and eight stages with anti-B. The yield in both cases was 2–3 drops of a very dilute suspension. When anti-B was added to free cells isolated with anti-A, or when anti-A was added to free cells isolated with anti-B, agglutination was essentially complete; that is, indistinguishable from the agglutination of unselected AB cells. The proportion of free cells estimated from the hemocytometer counts and the volumes at the beginning and end of the experiments was about 5×10^{-4} in each case, but these estimates were considered to be very unreliable. Moreover, it was uncertain whether the agglutinogen was actually absent from the cells. McKerns and Denstedt ('50) had found that cells remaining free after an agglutination with high-titer high-avidity antibody were agglutinated when fresh cells were added. They suggested that the free cells did not

lack agglutinogen, but were fully coated with agglutinin. Goudie ('57) reported the isolation of apparently B cells from AB blood by means of human anti-A. These cells gave a negative Coombs' test, suggesting that no agglutinin had been bound. According to Haberman, however, rabbit antihuman globulin, although fully standardized for anti-Rh, is quite unreliable as a Coombs' reagent for anti-A (Haberman and Aguilar, '57).

When the work was resumed in September of 1957, we explored methods of purifying exceptional cells in large quantities with lima bean anti-A lectin. Inagglutinable O cells labeled with Cr^{51} were added in small proportion to A or AB cell suspensions to trace the distribution of inagglutinable cells through various trial procedures. The inagglutinable cells were not entrapped if the clumps were allowed to settle by gravity alone. With centrifugation, however, only 30–40% of the inagglutinable cells could be freed from pellets formed from such mixtures in the presence of agglutinin. Cells were concentrated with minimal entrapment of the inagglutinable fraction in a lucite cylinder spun on its axis and lined with Kel-F fluorocarbon oil of density 1.9. The cells are deposited in a thin friable layer, and supernatant may be removed with a suction tube while the device is spinning. When the cylinder comes to rest, the sheet of cells breaks up and inagglutinable cells are efficiently freed.

Feasible procedures having been found, exceptional cells were isolated from seven different individuals. The washed cells from a pint of blood were placed in a large developing pan with 300–400 ml of saline and 200 ml of lima bean extract previously absorbed with B cells and diluted to a titer of 1:128. After massive agglutination, the mixture was poured into a separatory funnel and allowed to settle 10–15 minutes. The agglutinated mass was delivered through the stopcock and the supernatant (200–300 ml) was concentrated to about 50 ml in the cylindrical centrifuge, in batches of 30–40 ml. The subsequent separations were done over Kel-F in centrifuge tubes. The thin, disc-shaped pellets at the oil interface

were easily broken into a mixture of free cells and small flakes, and separation was effected by a brief low-speed centrifugation. Four to six such separations in undiluted lectin were required to purify inagglutinable cells. All procedures were carried out at 4°C. to increase the effectiveness of the phytoagglutinin. A pint of blood yielded 2–5 ml of a 1% suspension of inagglutinable cells. The proportion of inagglutinable cells lost during purification is unknown.

CHARACTERIZATION OF EXCEPTIONAL CELLS

To characterize the phenotype of the exceptional cells, we tested them for agglutination with a variety of serological reagents. In particular, we sought reagents that would agglutinate the exceptional cells more strongly than the unselected, and as will be seen, these were found. After purification, the exceptional cells were thoroughly washed and resuspended in saline. For each test, 2 drops of antiserum and 1 drop of cell suspension were centrifuged in a small test tube. Pellets were dislodged by tapping the tube and scored as follows: pellet unbroken, 4; two or three pieces, 3; many fragments, 2; very small fragments only, 1; complete resuspension, 0. Those that easily resuspended were examined for microscopic clumps. For direct comparisons, unselected cells from the same individual were always tested at the same time.

Table 1 gives the results of parallel tests on unselected and exceptional cells from one A_2B and four A_1B donors. The C.D.S. (Certified Donor Service) reagents are commercially available typing sera. The *Ulex* anti-H is a purified lectin from the seeds of *Ulex europeus,* available from Hyland Laboratories. *Dolichos biflorus* and *Vicia graminea* seed extracts were prepared in our laboratory, as were the B and O plasmas and absorbed lima bean extract.

The test with lima bean anti-A is an index of the degree of purity of the exceptional cells, and is generally satisfactory, although in two cases complete purification was not achieved.

TABLE 1

Reactions of exceptional cells in AB individuals

	A_1B								A_2B	
	K.A.		J.O.		M.L.		E.P.		L.R.	
	EC[a]	U	EC	U	EC	U	EC	U	EC	U
Anti-A reagents: Lima bean	0	4	0	4	0	4	±	4	0(+)	1
C.D.S. anti-A	0(++)	4	0(+)	4	0(+)	4	±	3	0(+)	2
C.D.S. anti-A_1	0	3	0	1	0(+)	1	1	1	0	0
Dolichos anti-A_1	0	4	0	4	0	3	±	3	0	0
B plasmas: E.D.	0(+)	4	0(+)	4	0(++)	4	3	4	0(+)	2
P.S.	1	4	0(+)	3	1	4	3	4	2	3
F.C.	0(+)	4	0(+)	3	0(+)	3	2	3	0(+)	+
O plasma absorbed with E.D. cells	2	4	3	4	2	4	2	3	3	4
reabsorbed with P.S.	2	4	0(+)	4	1	4	2	3	3	3
reabsorbed with A_2 cells	1	4	0(+)	4	2	2	2	2	0(+)	0(+)
Lima bean antibody eluted from A_2 cells	0	4	0	4	0	4	0	4	0	1
C.D.S. anti-B	4	4	4	4	4	4	4	4	4	4
Hyland *Ulex* anti-H	3	0(+)	2	1	2	0(+)	1	0	2	2
C.D.S. eel serum anti-H	3	2	4	1	3	2	1	1	3	3
C.D.S. anti-M	0	0	3	3	3	3	0(+)	0(+)	2	2
C.D.S. anti-N	3	3	3	3	3	3	3	3	3	3
Vicia graminea anti-N	4	4	4	4	4	4	3	4	4	4

[a] EC, exceptional cells selected with lima bean agglutinin; U, unselected cells. Symbols in parentheses indicate microscopic clumping.

Reactions with anti-B indicate that the exceptional cells from all donors were phenotypically B, as would be expected if the EC specifically lack the A agglutinogen. The MN typing again rules out a nonspecific failure to agglutinate, since EC are the same as unselected cells in this respect. Anti-H reagents reveal the most significant phenotypic modification of EC: loss of A_1 is accompanied by a great increase in H. Similarity of exceptional and unselected cells of the A_2B individual is to be expected, since anti-H reagents are ordinarily cross reactive with A_2. Significance of the increased reaction with anti-H as a clue to the origin of EC will be discussed later. Some of the anti-A reagents other than the lectin used in the isolations gave weak or partial agglutination of EC. In contrast to anti-A, this reaction is not seen with anti-A_1, except where it can be ascribed to impurity of the EC preparations. Reactions with C.D.S. anti-A were clearly partial, and a further separation yielding cells completely negative with C.D.S. anti-A was easily effected on EC from donor K.A. Such cells are not A_2, since A_2 cells are effectively removed by the phytoagglutinin. Differences in strength of the agglutinins offer no explanation, since the titer of the phytoagglutinin was higher than that of C.D.S. anti-A against the unselected cells of every donor tested. This suggests that in a fraction of the EC, differing with the individual, A is not completely absent, but modified in a manner corresponding to a weak subgroup of A. Should such modifications be caused by mutation, it is reasonable to surmise that the same mutation would occasionally occur in the germ line, and hence a search might reveal individuals showing negative reactions with lima bean lectin but positive with human anti-A.

Similar results were obtained with EC isolated from an A_1 and an A_2 (table 2). These exceptional cells were phenotypically indistinguishable from O cells, as would be expected in the absence of A agglutinogen. Some difficulty was experienced in isolating EC from donor J. L. In the end stages of

isolation the cells appeared to be sticky although not actually agglutinable. Very gentle tapping broke the pellet into clumps, but a more vigorous agitation easily dispersed these into free cells. A similar behavior had been noted with A_1B donor, E.P. It will be seen later that, in neither case, can the difficulty in isolation be attributed to a scarcity of EC.

TABLE 2

Reactions of exceptional cells from A_1 and A_2 selected with lima bean agglutinin

	J.L., A_1		T.W., A_2	
	EC[a]	U	EC	U
Lima bean anti-A	±	3	0	4
C.D.S., anti-A	±	3	±	2
Dolichos anti-A_1	0	4	0	0
Hyland *Ulex* anti-H	3	±	3	4
C.D.S. eel serum	4	3	4	3
C.D.S. anti-B	0	0	0	0

[a] EC, exceptional cells; U, unselected cells.

ANOMALOUS REACTIONS OF EXCEPTIONAL CELLS WITH HUMAN PLASMAS

The reactions of EC with B plasmas and absorbed O plasma in table 1 were at first taken as further evidence of modification of A to a state comparable to a subgroup. This view was brought into serious question, however, when EC were found to be agglutinable by AB plasmas, including that of the donor from which the cells were isolated. If the EC are agglutinated by plasmas having no anti-A, their reactions with B and O plasmas might also be quite unrelated to the anti-A content. The anomalous agglutinations were enhanced at low temperatures and weak or absent at 37°C. The first clue to their nature was the observation that agglutination by AB plasma was inhibited in the presence of lima bean extract. The inhibitory action was undiminished by boiling the extract or by complete absorption of its anti-A activity. Thus the inhibitor seems to be a heat-stable substance present in the crude bean extract. Agglutination of EC by B plasma was

also inhibited by bean extract, suggesting that the agglutination is largely spurious; that is, not caused by the anti-A activity of the B plasma. Since bean extract failed to inhibit partial agglutinations of EC with C. D. S. anti-A, these may, at least in part, have been valid manifestations of anti-A activity. When O cells were suspended in bean extract for 24 hours, then thoroughly washed, they became agglutinable by AB and other plasmas. They also exhibited the sticky behavior (before washing) previously seen in EC during two of the isolations. A plausible explanation is that the bean extract contains a substance that is chemically unrelated to its agglutinin and capable of rendering cells panagglutinable by human plasma. The EC become coated with this substance during isolation, to a greater or lesser degree depending on the time they are in contact with bean extract. An excess of the substance present in solution inhibits the panagglutination. In short, the anomalous plasma reactions are artifacts in the context of the present studies. Fortunately, they do not vitiate the principal results.

THE FREQUENCY OF EXCEPTIONAL CELLS IN DIFFERENT INDIVIDUALS

Isolation of inagglutinable cells in pure form is an unsatisfactory way of estimating their proportion in the individual. A more effective method was therefore devised, modeled after the isotope dilution methods widely used by chemists. About 5 ml of cells is suspended in saline with radioactive chromate. After labeling, the cells are thoroughly washed, agglutinated in a pan, and removed to a separatory funnel. A boundary forms after a few minutes of settling, and the agglutinated mass is separated from the supernatant. Ten to 20% of the cells, and hence of the radioactivity, remain in the supernatant. Most of these cells are agglutinable — they do agglutinate if centrifuged — but their concentration has been so reduced by the settling out of clumps that the rate of agglutination has become negligible. The cell concentration is there-

fore restored by the addition of unlabeled cells of the same type, referred to as carrier. Agglutination is repeated in a pan and followed by a second separation in a funnel. Successive agglutinations and separations are continued until the activity of the supernatant becomes constant. In addition to carrier, antibody is added at each stage so that the reaction mixtures have a fairly uniform composition. Dilution of the inagglutinable cells by these additions must be taken into account, and is the cumulative product of the dilutions incurred

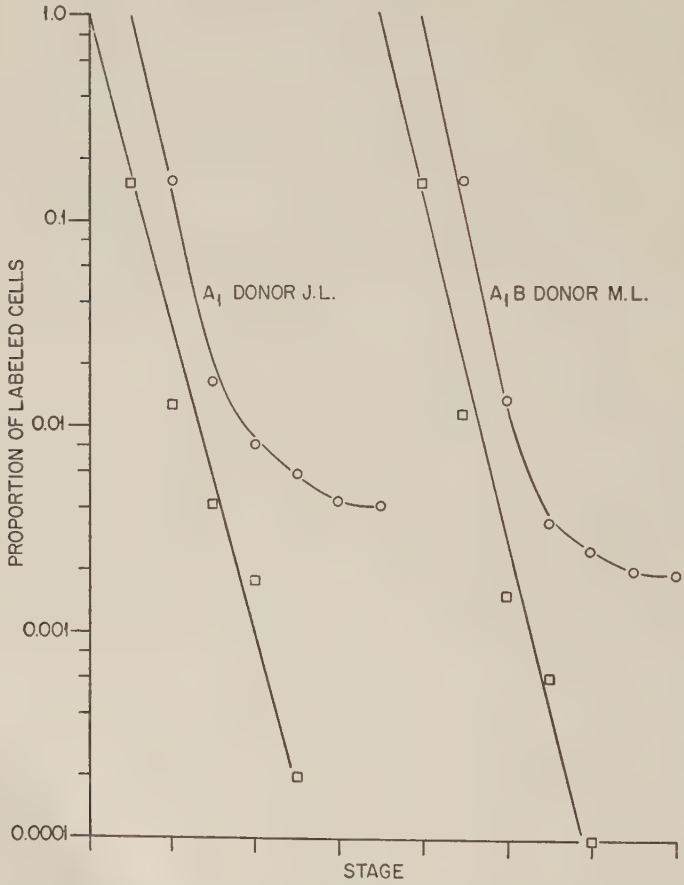

Fig. 1 Isotope dilution experiments. The proportion of labeled cells is plotted against the stage, or number of agglutinations with unlabeled carrier cells. Circles are experimental points; squares are the points replotted after subtraction of the value at stage six.

at successive stages. When the corrected activity becomes constant, all the agglutinable cells from the initial batch have been removed and only the inagglutinable remain. The ratio of final to initial activity then gives the proportion of inagglutinable cells.

TABLE 3

Proportions of cells inagglutinable by lima bean lectin, estimated by isotope dilution method

A_1B			A_2B		
Donor	Age	Proportion	Donor	Age	Proportion
	yr	*cells/10³*		*yr*	*cells/10³*
K.A. ♂	36	1.4	L.R. ♀	31	5.8
M.G. ♀	37	0.5			
M.L. ♀	37	1.9			
E.P. ♂	40	0.7			
J.O. ♀	39	0.7			

A_1			A_2		
Donor	Age	Proportion	Donor	Age	Proportion
	yr	*cells/10³*		*yr*	*cells/10³*
J.L. ♂	28	4.0	T.W. ♀	45	10.9
S.W. ♂	29	0.8			
H.A. ♂	27	1.3			
M.P. ♀	23	2.0			
N.A. ♂	39	2.0			

The approach to constant activity is shown in figure 1, where the ratio of the activity at each stage, corrected for dilution, to the initial activity, is plotted semilogarithmically. The curves shown indicate EC levels of 0.004 and 0.002. If it is assumed that the final point in each case represents EC alone, then the behavior of agglutinable cells alone can be plotted by subtraction of the EC from all the previous points. This plot is shown adjacent to each of the experimental curves. Its form is approximately exponential, suggesting that the agglutinable cells are being removed with about equal efficiency — within, say, a factor of two — at all stages. This suggests that the cell population is sharply divisible into two classes, agglutinable and unagglutinable, and argues against

the notion that the cells are widely distributed with respect to agglutinability. A reliance on such kinetic analysis is premature, but it may prove useful when better precision of the method is achieved.

The proportions of EC, determined by the isotope dilution method, in twelve individuals are shown in table 3. No correlations with age, sex, or group can be distinguished, except perhaps that A_2 and A_2B have the highest proportions. It is noteworthy that donors E.P. and J.L. do not show unusually low proportions of EC, although these were the most difficult to obtain by direct isolation.

NONGENETIC ORIGINS OF EXCEPTIONAL CELLS

We may first consider the hypothesis that all the cells are phenotypically alike *in vivo*. According to this view, the EC are manufactured during the experimental procedures. A variety of means is imaginable whereby the cell might be so altered as to superficially resemble a genuine preexisting variant. The possibility that the A agglutinogen might be mechanically removed from the cell surface during fragmentation of aggregates was examined by Goudie ('57). He was unable to produce inagglutinable cells in this manner. Complete saturation of the cell surface with irreversibly bound antibody was suggested by McKerns and Denstedt ('50) to explain the existence of free cells, and the reality of the phenomenon under their experimental conditions is backed by convincing evidence. Cells so affected would adhere to incompletely covered cells, but not to one another. In the isotope dilution method, such cells would therefore be removed by the fresh cells added to the system. In ordinary isolations, however, the phenomenon might cause serious confusion. It has probably not interfered with the present experiments because: (1) the yields in isolations are always lower than the corresponding proportions of EC given by the isotope dilution method, and (2) the isolated EC are more agglutinable by anti-H than the unselected cells. If some of the cells became

irreversibly coated with monovalent or blocking antibody, they would interfere with the isotope dilution determinations as well as with direct isolation. It is difficult to imagine, however, how such coating could augment the agglutinability with anti-H. The heat-stable agent in bean extract has no inhibitory effects on anti-H with EC. It is therefore highly unlikely that the increased H reactivity is itself an artifact produced by this agent. The H phenotype of isolated EC thus strengthens the validity of the isotope dilution method, which, in turn, confirms the *a priori* existence of the EC obtained by isolation.

A second hypothesis is that the phenotypic variants are present *in vivo* but are mainly independent of any genetic heterogeneity of the erythropoietic tissue. The distribution of numbers of agglutinogen sites per cell might be such that extreme variants are present even in a genetically uniform population. The transitory absence of an agglutinogen might be a normal feature of the erythrocyte life cycle. Such possibilities have not been excluded, although they are surely vulnerable to experiment. The kinetics of isotope dilution experiments do not favor a broad range of agglutinability; that is to say, a broad range of probabilities of removal of cells from the system. We do not know, however, how such probabilities are related to the number of agglutinogen sites. The increased H reactivity of EC would be a telling argument in favor of their genetic origin if the production of H substance by the O genotype were independent of the production of A substance by A genotypes. The existence of the rare Bombay group (Ceppellini, '52; Bhende *et al.*, '52; Levine *et al.*, '55) renders plausible, however, the role of H as precursor of A and B. The Bombay type is lacking in all three agglutinogens, H, A, and B, and the serum con-

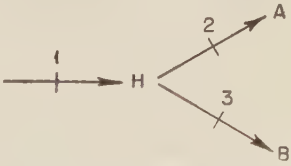

Fig. 2 Possible relation among H, A, and B substances in erythrocytes. The numbers represent positions of genetic blocks. The Bombay type is blocked at 1; O at 2 and 3; A at 3; B at 2; and AB nowhere blocked.

tains the three corresponding isoagglutinins. Although typed as O by the usual methods, people of this group can transmit B, and presumably A, to their progeny. It seems that persons lacking in H cannot produce A or B. The state of affairs can be explained by the schema in figure 2, suggested by Ceppellini. Since a lowered expressivity of A may well imply an accumulation of H, we cannot be sure that EC are genetically blocked at position 2, but if they are phenocopies, they are, at least, accurate facsimiles of the genuine mutants.

GENETIC ORIGINS OF EXCEPTIONAL CELLS

The expected effects of somatic mutation on the erythrocyte population can be understood only with reference to some model of the erythropoietic system. The most generalized model is not derived from bone marrow cytology, which is subject to varied interpretations, but rather from the elementary fact that the system remains in an approximately steady state when once established. This requires existence of a class of cells that have a positive probability of being the progenitors of cells present at any future time, no matter how remote. These may be regarded as stem cells, in contradistinction to other cells whose progeny will inevitably be absent from the system within a finite time. In the steady state, with constant mutation rate and no selection, the proportion of mutant stem cells must increase linearly with time; that is, the probability that progeny exist at a remote time is the same for mutant as for nonmutant stem cells, so that the only factor tending to change the frequency of mutants is the mutation rate itself. This state of affairs is analogous to a chemostat, with the stem cells representing the regulated population in the growth chamber, and the erythrocytes representing the overflow. On the other hand, mutations occurring in the cells that intervene between the stem lines and the erythrocytes will contribute a proportion of mutant erythrocytes that does not increase with age. If the mutation rate is assumed to be the same in stem cells as in intervening cells,

then the time-independent proportion of mutant erythrocytes will be exceeded by that of mutant stem cells within a small fraction of the normal human life span. To illustrate this, assume a mutation rate, a, per cell division. When a population of N cells is attained by clonal growth, the average number of mutants is $\bar{r} = aN \ln N$ (Lea and Coulson, '49). Since erythrocytes of the order of 10^{13} are present per individual, an average of 30 mutants would result from each mutation if this population were grown from a single cell; thus the proportion would average $30a$. The same proportion of mutant stem cells would be produced in 30 divisions, and since stem cells must divide, on the average, once every erythrocyte lifetime (100–120 days), this would require less than 10 years. If stem lines accidentally terminate, others must branch to maintain the steady state. It is reasonable to assume that the stem cell population is large enough to withstand a depletion of several orders of magnitude without noticeable disturbance of the proportion of mutants upon return to the normal population size. Mutations occurring prior to establishment of the steady state, as in the fetus, would lead to a large variance of samples from very young individuals, but this initial variance would soon be obscured as the age-dependent component increased. In summation, if the EC originate by mutation, their proportion at a given time will comprise age-dependent and age-independent components, the ratio of the components depending on the relative mutation rates in stem cells and intervening cells, and to a lesser extent on the relative sizes of the two populations.

MUTATION RATES

The mutation rate necessary to produce a typical proportion of mutants — say, 10^{-3} at age 35 — can be approximated if we ignore the age-independent component. Since the elapsed time is about 3×10^5 hours, a mutation rate of about 3×10^{-9} per hour would produce the observed value. If the rate is constant per time unit rather than per cell division, the age-

independent component will obviously be negligible. If mutation occurs only at division, the required rate would be about 7×10^{-6} per cell division (assuming a 100-day erythrocyte lifetime) uncorrected for an age-independent component that may be significant. The data do not warrant a more elaborate treatment. In table 3, the variation among individuals is greater than can be explained by the variance introduced during early development, unless the embryonic mutation rates are much higher than the adult. The alternatives would appear to be: (1) the process is not mainly mutational; (2) the mutation rate varies with the individual; or (3) the age-independent component is unexpectedly large, owing perhaps to a higher mutation rate in the intervening cells than in stem cells.

SOMATIC CROSSING OVER

If the variant cells in AB blood arise from mitotic crossovers, as suggested by Goudie ('57), they would exhibit the phenotype of homozygous B, whereas point mutation of A to O would yield heterozygous B cells. The crossover types would accumulate in the stem lines in the same manner as point mutants. It is possible that the reaction of EC with anti-H has some bearing on this mode of origin, since heterozygous and homozygous B cells may, in the future, prove to be distinguishable by anti-H. The reason for anticipating this is that A_1B bloods show the least reaction with anti-H, A_1 bloods are variable in this respect, and B bloods react fairly strongly. This suggests that the B allele does not of itself produce an agglutinogen that cross-reacts strongly with anti-H. Since the great majority of B individuals are heterozygotes, it is a plausible surmise that their H phenotype is determined mainly by the presence of the O allele. In short, one would expect homozygous B cells to possess no more H than A_1B cells. No proven B homozygote was encountered in this study, but if the foregoing conjecture is borne out it will indicate that the EC cannot, in the main, be attributed to mitotic crossing over.

EXCEPTIONAL CELLS IN A HOMOZYGOTES

Since the EC in AB individuals are phenotypically B, it follows (on the mutational hypothesis) that the equivalent cells in A homozygotes should be phenotypically A. The proportion of detectable exceptions should be negligible in homozygotes relative to heterozygotes. This is a *sine qua non* of genetic hypotheses of origin, but unfortunately it is not entirely inconsistent with a phenocopy hypothesis. On the simplest assumptions, the EC in the homozygote would be derived from a population of heterozygous cells produced at twice the usual rate owing to the presence of two A genes per cell. The average proportion of these that has existed up to a given time, however, is only half the proportion present at the end of that time. A homozygote would therefore have the square of the proportion of EC that would have been produced in an equivalent heterozygote.

It is reasonable to suppose that incomplete development of the phenotype might be more frequent in heterozygotes than homozygotes, but the relative frequencies of phenocopies need not necessarily coincide with those predicted for mutants. Since A homozygotes are frequent, the question of their EC content cannot long remain unsettled, but our only attempt to investigate this point gave an equivocal result. Dr. H. Gershowitz of the University of Michigan kindly supplied blood from two A parents of thirteen A children. The probability that at least one of the parents is a homozygote is 0.976. The isotope dilution experiments on these samples are shown in figure 3. Taken at face value, they almost exclude the mutational hypothesis, but unfortunately the five stages were insufficient to establish constant activity, and the meaning of the trend between stages three and five for Julia D. is uncertain. Small amounts of inagglutinable debris, perhaps certain white cells, might be labeled and produce a false equivalence of stages. This has been checked in subsequent experiments (with different donors) by lysis of the samples, and found not to occur. More than 80% of the activity was

released into solution on lysis, just as it is when fresh labeled erythrocytes are lysed. These experiments had reached constancy, however, at a much higher level than in the present case. It is also possible that the terminal trend of Julia D. was not an approach to an inagglutinable fraction, but to a new slope representing a fraction of the population somewhat less agglutinable than the majority.

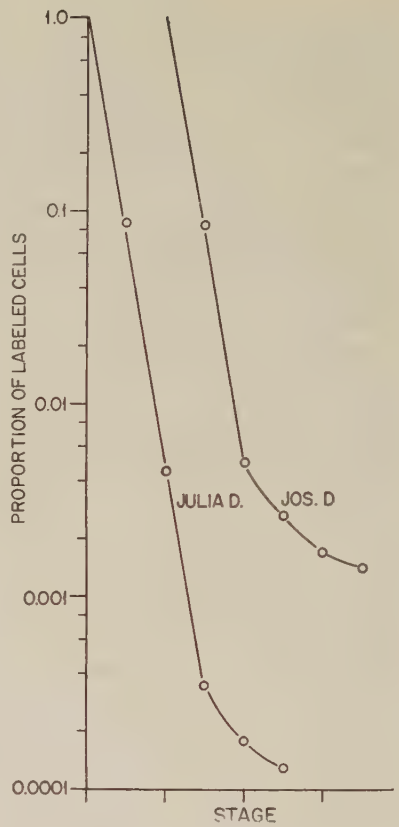

Fig. 3 Isotope dilution curves on A parents of 13 A progeny. Julia D. is a possible homozygote.

PROSPECTS

Although the preliminary nature of the experiments reported here need hardly be emphasized, they have indicated that phenotypic exceptions among the erythrocytes regularly occur and that it is practicable to enumerate and characterize them. Clarification of the relation to homozygosis and to the age of the individual is simply a matter of further experiments. In view of the variability already apparent among adults of similar age, examination of very young individuals, perhaps of umbilical cord blood, is most likely to reveal an age correlation provided agglutinogens can be used that are fully developed in the newborn. The value of extending the studies to other loci lies in the possibility that phenocopy production may be negligible with respect to some agglutinogens, and in the possibility of analyzing patterns of variation in more complex loci. Comparison of monozygotic twins

may reveal the degree to which the frequency of variants depends on early chance events. Mutagenic effects may be looked for in patients that have received heavy doses of radiation to the bone marrow, as in P^{32} treatment of polycythemia. Events in the stem cells and intervening cells would be distinguishable in such cases according to whether the effect is permanent or transitory. Finally, the direct visualization of bound agglutinin by autoradiography may reveal features of the distribution of cell phenotypes that are inaccessible to the techniques that have been used here. It must be confessed that our interest in erythrocyte agglutinogens is in reality a compromise with technical feasibility; we surmise that the genetics of the soma, although now limited in its approaches, will eventually prove equal in scope to that of the germ line.

OPEN DISCUSSION

(Papers by C. W. Cotterman and K. C. Atwood)

HEIDELBERGER [2]: Dr. Atwood, did you run through a curve of this sort with the human anti-A?

ATWOOD: No, I have isolated some cells with human anti-A, and they seem to be the same as the others, but I have not run through the curve with it.

HEIDELBERGER: I am a little worried about it because, using the bean anti-A, you are working with a cross reaction and not with true anti-A, and cross reactions rarely go to completion. I wonder whether you have a small fraction left over, and at least in the cases in which the human anti-A agglutinated the residual cells that were inagglutinable in the cross reaction this might seem a legitimate interpretation.

ATWOOD: Human anti-A gave only microscopic agglutination at best.

HEIDELBERGER: It was probably a true agglutination anyway.

[2] Michael Heidelberger, Rutgers University.

ATWOOD: I have no doubt it was. That means that if we had found two per thousand with the phytoagglutinin, then with human anti-A we would have found one per thousand, perhaps.

HEIDELBERGER: Or less than that. [Added note: Dr. Atwood later stated privately that the avidity of bean anti-A is greater than that of natural human anti-A. This lessens the validity of my comment, but calls attention to an interesting situation.]

BARIGOZZI[3]: Dr. Cotterman mentioned that somatic variation can indeed be induced by chromosomal rearrangement. I did not understand clearly whether he really means that somatic mutation is one of the most important sources of cell variation, and whether he disregards the germ-line mutations as the origin of the possible changes in phenotype. In *Drosophila,* we have very different manifestations of the same gene; these can become dominant instead of recessive in the heterozygous condition only because a chromosome section has been dislocated and brought close to heterochromatin after a germ-line mutation. In this case there is a quite variable manifestation probably caused, not by a somatic mutation, but by a mutation in one of the two gametes from which the individual is derived. The different manifestations are caused only by gene instability. The meaning of the term "gene instability" is open to question; certainly we know very little about it. Anyway, I think it is not entirely without interest for interpreting such complicated phenomena, to say that somatic mutation and germ-line rearrangements can be involved.

COTTERMAN: In making my statement in reference, not to blood mosaicism, but to mosaicism in general in plants and animals, I ventured to say that mutational mosaicism is perhaps a far more common process in nature than is chimerism. Under mutational mosaicism, I included all the following phenomena: somatic segregation, gene mutation, crossing over, chromosomal aberrations, and variegation mechanisms of several kinds. I do not know whether the last category

[3] Claudio Barigozzi, University of Milan.

would cover what you covered, but you see I was merely contrasting the rather bizarre mechanism of chimerism against other phenomena that might account for somatic mosaicism. I personally favor the idea that some sort of variegation, perhaps owing to heterochromatin effects, could account for the $O + A_2$ bloods that were mentioned.

ATWOOD: Dr. Strauss asked me whether we have any evidence on the question of somatic crossing over in the AB as being the source of exceptional cells; we really do not, but I think it is very unlikely. An analysis with reagents that distinguish subgroups might clarify the issue.

MEDAWAR[4]: I should like to make two points about these very interesting papers. The first is really only a comment about Dr. Cotterman's classification of the origin of chimeras. I think a new category needs to be added under Dr. Cotterman's heading B, and that is the type of chimera in which the host is an F_1 mouse, the term F_1 being used to mean a cross between two inbred mouse strains, and in which the donor of bone marrow is a mouse belonging to either of the parental strains or, of course, to the F_2, F_3, or F_4 progeny of a cross between them. The F_1 mouse is the universal recipient in this system of animals, so I think that just for completeness' sake this category should be added.

I want to ask one other question so elementary that the speakers may have regarded it as being hardly worth discussing. Red cells are a very highly heterogeneous population with regard to age. Some of them are only 3 days old and some of them are 3 months old. Is there any evidence that agglutinability changes as a function of the age of red cells?

ATWOOD: I know of no evidence for or against it.

COTTERMAN: I know of just a little evidence. The question of whether these aberrant or residual cells might represent some maturational stage differing from that of the agglutinable red cells has come up in connection with the free-cell phenomenon. In dealing with this minute fraction of negative

[4] P. B. Medawar, University College, London.

cells, various workers have estimated the proportion of reticulocytes present in these free cells to be not statistically different from that of the total cell population. This question reminds me of another point. I mentioned the J-positive cells in cattle, and you will recall that this antigen is evidently acquired by the erythrocyte from a soluble antigen in the plasma. Dr. Stormont informs me that if one cares to look for them one can find a small percentage of J-negative cells in the blood of J-positive cattle. He suspects these may be red cells that have just shortly before entered the circulation and have not had time to pick up their J.

Popp [5]: The fact that they do agglutinate with the anti-H of *Ulex* or eel serum indicates that, in this one case, they have not lost their ability to act with age and would therefore be some evidence against this.

Owen [6]: A point of possible relevance to your question, Dr. Medawar. If one studies the cells of donor type in an experiment in which bone marrow from another inbred strain of mice is injected into an irradiated host, one finds that this cell population rapidly becomes more agglutinable and more subject to hemolysis by specific antibody reagents than the cells of normal animals of the donor strain. It occurred to us that this might be the result of a relatively rapid turnover of cells in the strange and, perhaps hostile, environment in which these cells find themselves and that we were dealing here with a population of cells relatively young, relatively homogeneous in age, and relatively reactive. This, however, now seems to be an unlikely explanation for the excessive reactivity of these cells, because isologous bone marrow transplants do not at any time display this phenomenon, even though the red cell population derived from them must also pass through a period when a wave of young cells is liberated into the circulation.

Masouredis [7]: Dr. Atwood, do you think that inagglutinability of the exceptional cells may be caused by the presence

[5] R. A. Popp, Oak Ridge National Laboratory.

[6] R. D. Owen, Oak Ridge National Laboratory.

[7] S. P. Masouredis, University of Pittsburgh Medical School.

of incomplete, univalent, or blocking antibodies in the bean extracts? If such components are present and capable of sensitizing the red cells, the ability of such cells to agglutinate would be impaired.

ATWOOD: These plant extracts are said to contain large amounts of univalent antibody, but I do not think that is the thing that prevents the final free cells from agglutinating. It is hard to see how a coating of incomplete anti-A would make them react better with *Ulex* and eel serum.

MASOUREDIS: Of course, these sites could be elsewhere than H sites.

ATWOOD: An artificial increase in H sites seems unlikely wherever they are located.

HAUSCHKA [8]: There are some published data on mouse blood that are perhaps relevant to Dr. Medawar's question about possible effects of red cell age on agglutinability. P. A. Gorer and also G. Hoecker have found that agglutinogen E, one of the many isoantigens determined by the *H-2* system of genes, is not yet present on the red cells of newborn mice, but is fully differentiated about 5 days after birth. If adult C57BL mice are bled repeatedly, the titer of antigen E rises significantly. D. B. Amos and associates conclude that young red cells recently derived from nucleated erythroblasts have more E, and that E decreases as these cells age. The absence of E from the erythrocytes of newborn mice does not conflict with the observation on recently formed red cells in adults, since the former precedes primary antigenic differentiation. We may have here, then, a relation between the age of individual red cells and the strength of certain gene-determined agglutinogens on their surfaces.

HOECKER [9]: I should like to comment on Professor Medawar's remarks. In mice, *H-2* antigens are present in both blood and tissue cells. A study of these antigens through development done by O. Pizarro and P. Rubinstein showed

[8] T. S. Hauschka, Roswell Park Memorial Institute.

[9] Gustavo Hoecker, University of Chile.

that these antigens are not present at birth but appear about the third day of life. At this time, red blood cells react at a low titer. The titer increases thereafter until, by the fifth day, it is about the same as that of adult red blood cells. Absorption of antibodies by liver cells shows the same pattern: liver from 1- and 2-day-old mice does not absorb anti-*H-2* antibodies. At the third day, it absorbs a little, and by the fifth day of life it absorbs as much anti-*H-2* antibodies as adult liver. It seems, therefore, that age of the cells might have something to do with what we may call "maturation" of the antigens.

Briles [10]: In chickens, there are two blood group systems in which the time of appearance of the red cell antigens has been studied. Red cells genetically capable of bearing the A blood group antigens become fully agglutinable between the second and third day of embryonic development. Cells bearing the B agglutinogens, in general, become weakly agglutinable at the end of the first week of incubation, gradually increasing in titer until 1 or 2 weeks after hatching. The ability to agglutinate cells possessing antigens of the B system between 1 and 2 weeks of incubation differs widely for individual antisera made against the same homologous antigen. The striking difference in the ability of different reagents to agglutinate red cells samples from the same chick certainly indicates general specificity differences between antisera. Furthermore, since these antisera all react identically with adult cells, their differential reaction with the embryonic chick cells suggests strongly that the red cell antigens of the B system are going through a maturation process during the incubation period.

Stern [11]: In your abstract, Dr. Atwood, you mentioned nondisjunction as a possibility. It would be interesting to know whether this can be excluded. We should not restrict ourselves to the mutation interpretation too early.

[10] W. E. Briles, DeKalb Agricultural Association, Inc.
[11] Curt Stern, University of California, Berkeley.

ATWOOD: I probably should have mentioned that a number of other processes might contribute to these exceptional cells that are genetic processes, not strictly mutation. If these processes are very frequent in the intervening cells, they would add a large proportion independent of age. One thing that makes this seem likely is that one can imagine various kinds of aneuploidy that would be lethal in stem cells or in a zygote but still viable if near the end of the clone; that is, the cell might still mature. But we have no way of dealing with this just now. I think when we know whether the frequencies are correlated with age — this means that we will have to tolerate the variance that we find among people of the same age and it will have to be done on a lot of people to see the correlation — we will be able to say more about it.

LEDERBERG [12]: Dr Snell, is there any evidence from the behavior of coisogenic-resistant lines of mice for the maternal fetal type of chimerism that Dr. Cotterman postulated? This might be revealed by discrepancies between reciprocal backcrosses of the F_1 by either parent line in their tolerance to the other parent line, or in deviations from expected segregation ratios in F_2.

SNELL [13]: Actually, I do not think our numbers are such as to give critical evidence at the present time.

OWEN: Is it not true, Dr. Snell, that in most of these cases where viability has been best in the F_1 with their moderators, one gets little evidence of transplantability of skin that might follow any high frequency of maternal fetal transfer of that point?

SNELL: I think that would be true. I do not think there is any evidence at the present time that would indicate it, but it is a pretty hard thing to rule out.

BURNET [14]: I am going to ask your permission to introduce a slightly different story. Shortly before I left Australia I had

[12] Joshua Lederberg, University of Wisconsin.
[13] G. D. Snell, Roscoe B. Jackson Memorial Laboratory.
[14] F. M. Burnet, Hall Research Institute.

some correspondence with Fraser and Short of the wool research laboratory in Sydney. Over the last 3 or 4 years, they have been developing some material that has not yet been published, but I think it would be of great interest to people concerned with somatic genetics. It is not particularly relevant to the two papers we have had, but may be worth mentioning here. This is with regard to abnormalities of fleece. In a very extensive survey, covering perhaps 20 million Australian sheep, about 20 sheep showed fleece mosaics. On certain areas of the skin, much longer fleece appeared than the normal short fleece. Fraser and Short are interested primarily in the physiology of the fleece, but from the point of view of somatic mutation, it is a particularly interesting situation because of the distribution of the areas of fleece that they have found.

I have the figures available if anybody is interested, but roughly speaking there was one sheep with 44% of the skin area covered with long wool, three sheep with 20–25%, and then a group of five with 7–10%. There is a clear suggestion of somatic mutation involving cells very early in the process of segmentation of the fertilized ovum, and occurring with a frequency per cell of the same order as would be expected for mutation, i.e., around 10^{-6} to 10^{-7}.

Rogers [15]: We have some evidence relating to age and to one form of somatic cell variation involving the lung that we arbitrarily call a neoplasm. Our problem was to find why animals get spontaneous tumors when they get old rather than developing them when they are young and their tissue cells are much more rapidly dividing. It is known, for example, that with a variety of carcinogens the number of tumors of the lung initiated by a single exposure of animals of varying age is a function of the growth rate at the time of exposure. In our attempt to cast light on this seeming paradox, we implanted normal lung tissue from late fetal or neonatal mice into the leg muscles of old and young animals of the same strain, and after

[15] Stanfield Rogers, University of Tennessee Memorial Research Center.

6–12 months examined the implants and the lungs of the recipients for tumors. As usual, the older recipients had tumors but the younger did not; or in implanted mice left for almost a year, the mice young when implanted had markedly fewer tumors than the older ones. The incidence of tumors in the implants of lung in these animals, however, was the same, when tumors occurred at all, in both the old and young recipients. It was evident that the determining factor is a function of the age of the tissue rather than the age of the mice. Parallel studies indicated that the biologic age of the tissue was a function of the number of cell divisions in the implanted cell families.

I wonder to what extent, as time goes on and as more data are accumulated, analogous results might be found in other forms of somatic cell variation. It would be interesting by your system to find, for example, the relative numbers of new sub-blood types in old and young individuals.

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HISTOCOMPATIBILITY CHANGES IN TUMORS

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ONE FIGURE

Intercellular variation of various biological, morphological, or cytochemical characteristics occurs frequently in populations of neoplastic cells. Some forms of variation result in stable, permanent, and irreversible differences, being suggestive of genetic changes (see reviews by Foulds, '54; Furth, '53; Hauschka, '57; Klein, '56; Law, '54a). By selective procedures, the growth of one cell type may often be favored at the expense of other types (Hauschka *et al.*, '56; Kaziwara, '54; Klein and Klein, '56a; Law, '54b; Loustalot *et al.*, '52). This may lead to a change in the behavior of the whole population of neoplastic cells. Characteristics thus altered may be related to malignancy in, e.g., experimentally induced shifts in invasiveness and metastasizability (Ringertz *et al.*, '57) and related surface characteristics (Purdom *et al.*, '58), ability to grow in the dissociated ascites form (Klein and Klein, '56a), and drug resistance (Law, '54a, b). It would be tempting to infer that most cases of tumor progression, i.e., the stepwise development shown by many different tumors on their way toward increased autonomy (Foulds, '54), are caused by the random occurrence of increasingly autonomous cellular variants and their selective overgrowth that replaces the former type. Some cases of tumor progression seem to be attributable to other mechanisms (Klein and Klein, '57), although variation and selection may be more usual.

To gain more insight into the problem of tumor progression, more information about the nature of intercellular variation

in populations of neoplastic cells is necessary; e.g., to what extent the variation can be considered as genetic; what types of variation occur and in what frequencies; how they differ in different categories of tumors and in different tumors of the same category; and if certain types of variation are amenable to experimental modification. Experimental approach is now greatly hampered by lack of suitable marker genes detectable in somatic cells. Selective systems are also needed for identification of small variant fractions in large cell populations.

Some years ago, in looking for a system that would satisfy these requirements, we undertook a study of tumor-cell populations *in vivo*. This choice was owing to the feeling that *in vitro* studies of neoplasia often suffer from various drawbacks because there is no host organism (Klein, '57). Treating tumor cells and other somatic cells as microorganisms in culture may reveal a great deal about their potentialities for independent evolution under controlled conditions. But when approaching the problem of neoplasia as it exists *in vivo,* we can hardly treat tumor cells so, since the most important aspect of their behavior concerns their relation to the superimposed mechanisms of homeostasis.

If consideration is restricted to *in vivo* systems, the use of *histocompatibility* (*H*) genes as markers provides an approach to studies of cellular variation in terms of gene products and may thus yield some information about the behavior of the genetic determinants themselves. But this will hold true only under some carefully selected conditions. Since the mouse appears to have at least fourteen *histocompatibility* genes (Snell, '53), variation should be restricted to *one H* locus. Snell's development ('48, '55) of isogenic-resistant (IR) lines of mice made this possible. Making use of neoplasms that have arisen in such animals, we can create situations in which a tumor's ability to grow in a certain host will depend on the allelic substitution of a single gene locus in the cells of the tumor and of the host. Such a situation might plausibly be used for selective detection of variant tumor cells that differ

from the original type with regard to the products of the particular gene locus in question.

We tested such a system, first in model experiments (Klein and Klein, '56b) and then on a series of methylcholanthrene-induced sarcomas (E. Klein *et al.*, '57). Similar systems were proposed by Lederberg ('56) and used by Mitchison ('56). We used tumors originating in F_1 mice produced by crossing two of Snell's IR lines differing in allelic substitution at the compound *H-2* (*histocompatibility-2*) locus, which is the strongest known barrier against homotransplantability of tissues and tumors in the mouse (Snell, '57). Since both parental lines have the same isogenic background, such hybrids are homozygous (at least theoretically) with respect to their entire genome, except *H-2* — in which the parental strains differ, making the hybrids heterozygous. As a rule, neoplasms that originate in hybrids of this type do not grow in either parental strain, since each parental strain can respond with a homograft reaction provoked by the isoantigenic products of the foreign *H-2* allele derived from the other parental strain and both alleles are present in the tumor cells. Such tumors can be maintained only by transplantation to F_1 animals having the same genetic constitution as the animal in which they arose. If, however, a specific mutation occurs at one of the two *H-2* loci in a tumor cell that proceeds in the direction of the noncorresponding parental type, or one that renders the gene isoantigenically inactive for the noncorresponding parental strain, this change could be expected to confer an absolute selective advantage on the bearer cell and its progeny, whenever the cell population is tested in that particular parental strain. This would hold true only if the homograft reaction that destroys the unchanged and incompatible cells did not also destroy the mutant in a nonspecific way. Our model experiments indicated that the latter possibility can be dismissed and that the prospects for demonstrating specific mutations in this system are favorable. Subsequently, we tested five methylcholanthrene-induced sarco-

mas in this system (E. Klein *et al.*, '57). In three of them, no variants capable of growth in any of the parental strains could be detected. One was nonspecific and grew without discrimination in both parental strains and in foreign genotypes. The fifth sarcoma, MSWB, regularly gave rise to a certain number of variant tumors that grew in one of the parental strains (A.SW) but never in the other (A). In later tests, these variants grew regularly in A.SW, even in hosts preimmunized with strain A tissue or with the original line of the MSWB tumor. Serological evidence indicated the loss of the isoantigenic components specifically determined by the $H\text{-}2^A$ factor, derived from the A strain. X irradiation increased the yield of variants.

Results reported in this paper represent a direct continuation of these studies. The 23 sarcomas studied were induced by methylcholanthrene in five different F_1 combinations of the A/Sn strain and its three coisogenic-resistant sublines, differing at the *H-2* locus. A number of transplantation tests were supplemented by serological analysis, and a selected group of tumors and their variants were examined with regard to their chromosome cytology by Dr. Klaus Bayreuther.

MATERIALS AND METHODS

Four IR lines of mice developed by Snell ('48, '55) were used, all with a strain A background and differing only at the *H-2* locus and its immediate surroundings. The original A/Sn line is genotypically $H\text{-}2^A H\text{-}2^A$; the other three sublines, A.SW, A.BY, and A.CA are $H\text{-}2^S H\text{-}2^S$, $H\text{-}2^B H\text{-}2^B$, and $H\text{-}2^F H\text{-}2^F$, respectively (Snell, '55, Allen, '55). The isoantigenic complex determined by each of these *H-2* alleles is denoted by a series of letters, each corresponding to an antigenic component, detectable by hemagglutination tests. Thus $H\text{-}2^A$ is antigenically CDEFKH, $H\text{-}2^S$ is CEFGS, $H\text{-}2^B$ is BEF, and $H\text{-}2^F$ is GHI (Hoecker, '56). The letters denote only those antigens for which serological evidence exists, and the possibility is by no means excluded that still other factors may be present.

The strains are maintained by continuous single-line, brother-to-sister mating in our laboratory. After each four or five generations, females of the sublines A.SW, A.BY, and A.CA are crossed with males of the A/Sn strain. F_2 from these crosses are subsequently challenged with a tumor of A/Sn origin, and the survivors are antigenically typed by the hemagglutination test of Gorer and Mikulska ('54). Mice showing the isoantigenic type of the particular IR subline entering the cross are subsequently used to replace the breeding pairs of this line, and their progeny is bred by brother-to-sister crossing for another four or five generations. This repeated backcrossing to the A/Sn strain was introduced to maintain the common genetic background as closely identical as possible in all four lines.

Animals 2 to 3 months old of both sexes were used for the transplantation experiments. All mice were earmarked at weaning, each genotype having a different mark; breeding mice were earmarked individually. A standard diet in pellet form and drinking water were available *ad libitum.*

Sarcomas were induced by injecting 1 mg of methylcholanthrene dissolved in trioctanoin in 0.1-ml volumes intramuscularly into the right thigh of 1-month-old F_1 mice from various combinations of the four strains. Three to 4 months later, a number of animals developed sarcomas. Tumors that showed no signs of ulceration were removed under aseptic conditions. One part of the tissue was fixed for histological examination, another part was frozen in our tumor bank, maintained at —79°C. (G. Klein *et al.,* '57), and the rest was tested by inoculation into mice of various genotypes. No tumors were carried serially over extended periods. The bank of frozen tissue enabled us to study the immunogenetic behavior of these neoplasms while they were still as close as possible to their original conditions. Tumors of the first transfer generation were usually frozen in several parallel tubes. For a test, one tube was thawed, its contents inoculated into mice of the original genotype, and the resultant tumors were

tested. Tests were continued until the supply of parallel tubes was exhausted. Then a series of tubes from the second transfer generation were frozen and used for further tests. In this way, accumulation of serial passages was delayed considerably and no tumors except MSWB were studied after more than five transfer generations. MSWB has been carried for a maximum of 11 transfers. We could find no evidence of any change in immunogenetic specificity of tissue frozen and thawed by our method (G. Klein *et al.*, '57), which does not involve addition of glycerin or artificial solutions of any kind to the solid piece of fresh tumor tissue.

Prior to inoculation, solid tumors aseptically removed were freed from macroscopically necrotic areas and brought into suspension by pressing them through a 60-mesh stainless steel screen into Ringer's solution. Roughly comparable amounts of suspended tissue were injected subcutaneously into the right flank or bilaterally. Developing tumors were followed by regular palpation. Mice killed by growing tumors were considered positive; those surviving for 3 months without tumor development, or whose tumor had regressed during this period, were registered as negative.

Usually not more than three to five mice of a given genotype were available for testing. Results of different tests were pooled for each tumor and genotype, unless there was reason to suspect that the different inocula did not represent a homogeneous sample. The final data given in the tables represent such pools unless otherwise stated.

In experiments where quantitative inocula were used, the rough tumor suspensions were filtered through a 100-mesh stainless steel screen and two layers of gauze. The final suspension was diluted with Tyrode's solution containing 0.5% eosin, as recommended by Schrek ('36). Cells not stained with eosin were rapidly counted in a hemocytometer, and measured aliquots containing known numbers of cells were inoculated.

For the serological experiments, antisera were obtained by repeated inoculations of tumor tissue indigenous to a certain mouse strain or a certain F_1, into IR mice carrying *H-2* alleles foreign to the tumor. Four to seven challenges were given at intervals of 10–15 days. The mice were bled from the retroorbital sinus through a glass capillary, 0.5–0.8 ml of blood from each animal. Blood pooled from two or three mice was placed in a small pyrex centrifuge tube, allowed to clot at room temperature, and centrifuged at $800 \times g$ for 15 minutes. The serum was lyophilized and stored until use.

A mixture of minced liver, kidney, and spleen, washed three times with Ringer's solution, and packed by centrifugation was used for antiserum absorption. Equal volumes of tissue and antiserum 1:4 were incubated at 37°C. for 1 hour. One or two absorptions were usually sufficient. After incubation the mixture was centrifuged and the absorbed antiserum collected.

The agglutinating capacity of the antisera was tested in the system containing dextran and human serum, described by Gorer and Mikulska ('54).

The dextran preparation used was Intradex, salt free (Glaxo), made up to 6% solution in 5% glucose. Human serum was prepared from blood collected under aseptic conditions from healthy donors. Samples of human serum were incubated at 56°C. for 1 hour, then absorbed with washed mouse tissue by the procedures described for antisera. Absorbed human sera were stored at —20°C.

Agglutination tests were as follows: Red cells, obtained by puncture of the retroorbital sinus, were washed twice in 0.85% saline and suspended to a 2% concentration in a 1:1 mixture of human serum and saline. Antiserum dilutions were made with dextran solutions, diluted to 2% with saline. Controls contained dextran, human serum, and mouse red cell suspensions, but no antiserum. The total volume in the agglutination tubes was 0.05 ml, consisting of 0.025 ml of diluted antiserum in dextran or, in the controls, dextran alone,

and 0.025 ml of erythrocyte suspension. Mixtures were incubated at 37°C. for 1 hour. Agglutination was read exactly as described by Gorer and Mikulska ('54).

RESULTS

Transplantation tests

Tumors originating in (A × A.SW)F_1. Seven tumors of this category were tested, and the results are summarized in table 1. All the original F_1 donors were derived from crosses between A/Sn males and A.SW females. Four tumors (MNSB, MNSC, MNSD, and MSWG) grew in only (A × A.SW)F_1 mice and were unable to grow in any of the parental strains, at least within the limits imposed by the number of animals available and other experimental conditions. One tumor (MSWB) studied in a previous work (E. Klein *et al.,* '57) grew in some recipients of one parental strain (A.SW) but never in the other (observed in the previous work and now confirmed on more-extensive material). Variant tumors selected in A.SW mice grew in 92% of A.SW recipients, whereas the original tumor (MSWB) grew in only 24%. A high percentage of these variants grew also in A.SW mice preimmunized against A tissue or against the original line of the tumor, whereas the MSWB tumors grew in only 2% of the preimmunized mice. Individual variants, however, differed in their ability to grow in preimmunized mice. Details of the studies on this tumor will be published elsewhere by K. Bayreuther and E. Klein. I shall only say here that the variants capable of growth in the A.SW parent remained entirely specific and grew in neither the other parental strain nor foreign genotypes. Only one variant line (MSWB-SY) showed incomplete specificity. This was recovered from an A.BY mouse challenged for the second time with an MSWB variant taken from an A.SW host. This tumor grew in a certain percentage of A, A.BY, and A.CA mice (table 1). When preimmunized against the original MSWB line, A, A.BY, and A.CA mice were nevertheless entirely refractory even to this variant,

TABLE 1

Transplantation tests with sarcomas induced by MC in (A ♂ × A.SW ♀)F_1 (genotype $H\text{-}2^AH\text{-}2^S$)

TUMOR	NO. OF MICE KILLED BY PROGRESSIVELY GROWING TUMORS/TOTAL NO. OF INOCULATED MICE IN INDICATED RECIPIENT STRAINS						
	(A × A.SW)F_1	A ($H\text{-}2^AH\text{-}2^A$)		A.SW ($H\text{-}2^SH\text{-}2^S$)		A.BY ($H\text{-}2^BH\text{-}2^B$)	A.CA ($H\text{-}2^FH\text{-}2^F$)
		Untreated	Immunized against A.SW	Untreated	Immunized against A		
DSWB	63/63	0/55	—	3/60	—	—	—
Variant DSWB-S taken from A.SW	7/7	0/14	—	26/29	0/10	—	—
MSWB	98/98	0/85	—	116/484	3/158	0/43	0/29
Pooled variants MSWB-S taken from various A.SW hosts	83/83	0/50	0/5	150/163	181/210	4/35	1/36
Variant MSWB-SY from A.BY mouse bearing MSWB-S	46/46	6/74	0/26	70/70	15/16	29/65 [a]	7/36
MNSB	32/32	0/40	—	0/33	—	0/5	—
MSWC	91/91	54/76	10/35	61/78	95/135	10/16	22/35
MNSC	17/17	0/24	—	0/27	—	—	—
MNSD	7/8	0/8	—	0/11	—	—	—
MSWG	8/8	0/12	—	0/15	—	—	—

[a] In A.BY immunized against MSWB, 0/40.

whereas all preimmunized A.SW mice died with growing tumors. Repeated attempts to produce sublines of this variant with an improved and selective ability to grow in A, A.BY, or A.CA by serial selective transfer failed entirely; only the ability to grow in A.SW consistently bred true.

A fifth tumor (DSWB) was highly specific but gave a few growing tumors in the A.SW parent (5%). These variants grew in 90% of the A.SW mice, but there were no takes in ten A.SW hosts preimmunized against the original line of the tumor.

Table 1 also includes the accumulated data obtained with the previously described MSWC sarcoma. This tumor grew in a certain percentage of all foreign genotypes tested, including hosts preimmunized against the original line, but never in all of any one genotype. In spite of repeated attempts, it was not possible to select sublines specific in their compatibility requirements from this tumor.

Taken as a whole, the results indicate that all true variants recovered from tumors of (A ♂ × A.SW ♀)F_1 origin represented changes in the direction of the A.SW parent.

Tumors originating in (A × A.CA)F_1. Six tumors that belong to this category were tested; all were induced in hybrids derived from crosses of A/Sn males with A.CA females (table 2). MACB, MACE, MACF, and MACG did not grow in all mice of the original (A × A.CA)F_1 genotype. This is reminiscent of the phenomenon described by Prehn and Main on similar material ('57). It seems peculiar that this effect was manifested only in this particular genotype. It was especially apparent with the MACG sarcoma.

One tumor (MACB) gave no variants in the parental strains; but only a few mice were used. Three tumors (MACE, MACF, and MACG) gave rise to a certain proportion of growing tumors in parental strain A.CA. Only variant MACG-C has been tested. It grew well in A.CA mice, including hosts preimmunized against tissue of strain A origin.

Two tumors (MACC and MACD) showed an interesting and mutually similar behavior. They gave rise to a certain num-

TABLE 2

Transplantation tests with sarcomas induced by MC in (A ♂ × A.CA ♀)F_1 (genotype $H\text{-}2^AH\text{-}2^F$)

TUMOR	NO. OF MICE KILLED BY PROGRESSIVELY GROWING TUMORS/TOTAL NO. OF INOCULATED MICE IN INDICATED RECIPIENT STRAINS						
	(A × A.CA)F_1	A ($H\text{-}2^AH\text{-}2^A$)		A.CA ($H\text{-}2^FH\text{-}2^F$)		A.SW ($H\text{-}2^SH\text{-}2^S$)	A.BY ($H\text{-}2^BH\text{-}2^B$)
		Untreated	Immunized against A.CA	Untreated	Immunized against A		
MACB	3/11	0/20	—	0/5	—	2/13	0/3
MACC	31/31	10/34	—	3/22	—	0/8	0/5
Variant MACC-A taken from A host	9/9	39/40	30/43	1/16	0/7	0/15	—
Variant MACC-C taken from A.CA host	7/7	1/20	—	22/22	26/26	0/3	0/3
MACD	5/5	1/10	—	1/5	—	0/10	0/9
Variant MACD-A taken from A host	2/2	19/21	9/19	0/5	0/6	0/2	0/3
Variant MACD-C taken from A.CA host	5/5	0/13	—	3/3	21/21	0/9	0/2
MACE	14/16	0/13	—	1/14	—	0/9	0/5
MACF	10/14	0/24	—	1/21	—	0/10	0/9
MACG	2/20	0/17	—	5/7	—	0/10	0/5
Variant MACG-C taken from A.CA host	2/7	0/4	—	4/4	12/14	—	—

ber of progressively growing tumors in *both* parental strains. On subsequent testing, these variants showed a surprisingly high degree of specificity for the particular parental strain in which they appeared and did not grow at all, or only slightly, in the opposite parental strain. When tested in immunized mice of the parental types that had been inoculated previously with normal or tumorous tissue containing the isoantigenic components of the opposite parent, a difference appeared between the two variant types. With both the MACC and MACD tumors, variants selected in the A.CA parent grew in all A.CA test mice preimmunized against A tissue. On the other hand, variants selected in the A strain grew in 70% (MACC-A) and 47% (MACD-A) of A mice preimmunized against A.CA tissue.

Taken as a whole, the results indicate that, although variants with selective compatibility did appear in both parental strains when we were testing tumors induced in the (A ♂ × A.CA ♀)F_1, there was a certain predilection for the A.CA strain, both in frequency of variants and their ability to grow in preimmunized hosts.

Tumors induced in (A × A.BY)F_1. Only two sarcomas of this category have been tested (table 3). They were induced in mice derived from crossing an A/Sn male with an A.BY female. MAYA has not given rise to any variants, but MAYB grew in a certain percentage of both parental strains. When tested subsequently, these variants behaved in a specific way and grew only in the corresponding parental strain; however, this observation is based on a small number of mice. Only the variant selected in the A.BY parental strain grew in all preimmunized mice (21/21); the variant selected in the A strain grew in only about half the A mice preimmunized against A.BY tissue (5/11).

Tumors induced in (A.SW × A.CA)F_1. Three sarcomas of this category were tested, all induced in F_1 mice from crosses of A.SW males with A.CA females (see table 4). One tumor (MCSB) was specific and grew in only the F_1 of the original type; another (MCSA) gave rise to a number of

TABLE 3

Transplantation tests with sarcomas induced by MC in (A ♂ × A.BY ♀)F_1 (genotype $H\text{-}2^A H\text{-}2^B$)

TUMOR	NO. OF MICE KILLED BY PROGRESSIVELY GROWING TUMORS/TOTAL NO. OF INOCULATED MICE IN INDICATED RECIPIENT STRAINS					
	(A × A.BY)F_1	A ($H\text{-}2^A H\text{-}2^A$)		A.BY ($H\text{-}2^B H\text{-}2^B$)		A.SW ($H\text{-}2^S H\text{-}2^S$)
		Untreated	Immunized against A.BY	Untreated	Immunized against A	
MAYA	23/23	0/41	—	0/38	—	1 [a]/9
MAYB	14/14	8/28	—	13/17	—	0/3
Variant MAYB-A taken from A host	—	8/8	5/11	0/6	—	—
Variant MAYB-Y taken from A.BY host	—	0/5	—	4/4	21/21	0/3

[a] Further testing of this tumor gave the following results: A, 0/3; A.SW, 0/3; (A.CA × A.BY)F_1, 2/3.

TABLE 4

Transplantation tests with sarcomas induced by MC in (A.SW ♂ × A.CA ♀)F_1 (genotype $H\text{-}2^S H\text{-}2^F$)

TUMOR	NO. OF MICE KILLED BY PROGRESSIVELY GROWING TUMORS/TOTAL NO. OF INOCULATED MICE IN INDICATED RECIPIENT STRAINS						
	(A.SW × A.CA)F_1	A.SW ($H\text{-}2^S H\text{-}2^S$)		A.CA ($H\text{-}2^F H\text{-}2^F$)		A ($H\text{-}2^A H\text{-}2^A$)	A.BY ($H\text{-}2^B H\text{-}2^B$)
		Untreated	Immunized against A.CA	Untreated	Immunized against A.SW		
MCSA	4/4	0/15	—	3/10	—	0/10	0/8
Variant MCSA-C taken from A.CA host	3/3	6/10	4/19	15/15	28/28	0/7	5/6
Variant MCSA-CS from A.SW mouse bearing MCSA-C	—	11/11	4/21	7/7	—	—	3/3
MCSB	12/12	0/26	—	0/21	—	0/5	0/9
MCSC	3/3	4/15	—	2/10	—	0/7	0/8
Variant MCSC-C taken from A.CA host	3/3	0/17	—	13/13	11/14	0/4	—
Variant MCSC-S taken from A.SW host	3/3	14/16	6/21	5/9	—	3/6	—

tumors in the maternal strain. When tested further, these variants, termed MCSA-C, grew in all A.CA mice, including those preimmunized against strain A tissue. They were not quite specific for A.CA, however, since they also grew in some A.SW and A.BY mice. An effort to select a variant specific for A.SW and less likely to grow in the other types has failed so far (e.g., MCSA-CS, table 4). The low frequency of growth in A.SW preimmunized by A.CA tissue (about 20%) could not be improved by selection.

The third sarcoma of this group (MCSC) grew in a certain proportion of both parental types. The tumors that have appeared in A.CA were specific for this strain and grew also in a large proportion of preimmunized hosts. Tumors taken from A.SW were much less specific; they did not grow in all untreated A.SW hosts; in preimmunized mice, only six tumors grew of 21 inoculated. This variant also gave rise to quite a high proportion of tumors in A.CA and in A.

As a whole, the data suggest that sarcomas derived from this hybrid combination may have a certain predilection for the maternal A.CA strain with respect to the formation of specific variants.

Tumors induced in (A.SW × A.BY)F_1. Five sarcomas of this category were tested (see table 5). Unfortunately, both types of reciprocal hybrids were available at the time of the methylcholanthrene injections and no distinction was made between them. It is therefore impossible to identify the paternal and maternal strains for the tumors of this particular hybrid type; they may vary for different tumors. With this reservation, the findings can be summarized as follows:

No tumors were entirely limited to the original F_1 type. Four sarcomas (MWYB, MYSA, MYSB, and MYSC) showed, nevertheless, a comparatively high degree of specificity. Besides in the original type, there were a few takes in one or both parental strains and no growth in mice bearing only foreign alleles at the *H-2* locus. It was unusual to find, however, that one tumor (MWYB) grew in all mice of the A.SW parental strain immediately and MYSB grew in one-half

TABLE 5

Transplantation tests with sarcomas induced by MC in (A.SW × A.BY)F_1 (genotype $H\text{-}2^S H\text{-}2^B$)

TUMOR	NO. OF MICE KILLED BY PROGRESSIVELY GROWING TUMORS/TOTAL NO. OF INOCULATED MICE IN INDICATED RECIPIENT STRAINS						
	(A.SW × A.BY)F_1	A.SW ($H\text{-}2^S H\text{-}2^S$)		A.BY ($H\text{-}2^B H\text{-}2^B$)		A ($H\text{-}2^A H\text{-}2^A$)	A.CA ($H\text{-}2^F H\text{-}2^F$)
		Untreated	Immunized against A.BY	Untreated	Immunized against A.SW		
MWYA	9/9	19/19	17/18	3/13	—	3/16	4/12 [a]
MWYB	10/10	13/13	—	0/17	—	0/12	0/9
MYSA	15/15	1/16	—	2/15	—	0/10	0/9
MYSB	5/5	5/10	—	0/5	—	0/11	0/5
Variant MYSB-S taken from A.SW host	—	5/5	20/28	1/7	3/16	1/3	—
Variant MYSB-SY taken from A.BY mouse bearing MYSB-S	—	3/3	3/5	4/5	3/4	—	—
MYSC	4/4	1/18	—	2/10	—	0/6	—
Variant MYSC-S taken from A.SW mouse bearing MYSC	—	3/3	15/22	2/2	9/11	—	—
Variant MYSC-Y taken from A.BY mouse bearing MYSC	—	3/3	13/20	6/6	15/17	0/3	0/4

[a] In A.CA preimmunized against A.SW, 0/7.

the mice of the same parental strain. There was a surprising lack of specificity of the variants selected from one or the other of the parental strains adequately tested (variants of MYSB and MYSC). Preimmunization of the test animals did not improve the situation to any considerable extent. On the basis of the present small amount of material, it is difficult to judge whether this behavior is characteristic of the individual tumors or represents a more general feature inherent to this particular hybrid combination.

One tumor (MWYA) exhibited a considerable lack of specificity from the beginning but showed a distinct preference for the A.SW parental strain. On the whole, the A.SW parental strain seems to have been a more frequent site of variant formation than A.BY, although the difference is less clear cut with this hybrid type than in the other cases described.

SUMMARY OF ALL TRANSPLANTATION TESTS

Results obtained with the different hybrid combinations are summarized in table 6. Twenty-three tumors were tested. Although the number of mice used for the different testings was quite variable, certain patterns of behavior nevertheless became apparent. Seven tumors were entirely restricted in their growth to the F_1 genotype of origin and gave no variants in either parental strain; four of these belonged to the $(A \times A.SW)F_1$ combination. Eight sarcomas grew in a certain proportion of *one* parental type only; some (such as DSWB and MSWB) were repeatedly tested in quite sizeable animal groups. They consistently refused to grow in the other parental type. As a rule, variants from one of the parental strains showed a high degree of specific compatibility with this strain upon subsequent testing, usually including the ability to grow in hosts preimmunized against the opposite parental strain. There were some exceptions to this rule, however; certain variants behaved in a less-specific way and grew to some extent in the opposite parental strain and also in for-

TABLE 6

Summary of transplantation tests

GENOTYPE OF ORIGIN	TUMOR		IN PATERNAL STRAIN			IN MATERNAL STRAIN		
	Name	Specificity	Percentage growth	Specificity of selected variants	Percentage growth of variants in preimmunized mice	Percentage growth	Specificity of selected variants	Percentage growth of variants in preimmunized mice
(A ♂ × A.SW ♀)F_1	DSWB	High	None (55)*	—	—	5 (60)*	Specific	None (10)*
	MSWB	High	None (85)	—	—	24 (484)	High in 18 samples tested; low in one	86 (210)
	MNSB	High	None (40)	—	—	None (33)	— Not specific	— 70
	MSWC	Very low	71 (76)	Not specific	29 (35)	78 (78)		(135)
	MNSC	High	None (24)	—	—	None (27)	—	—
	MNSD	High	None (8)	—	—	None (11)	—	—
	MSWG	High	None (12)	—	—	None (15)	—	—
(A ♂ × A.CA ♀)F_1	MACB	High	None (20)	—	—	None (5)	—	—
	MACC	High	29 (34)	Specific	70 (43)	14 (22)	Specific	100 (26)
	MACD	High	10 (10)	Specific	47 (19)	20 (5)	Specific	100 (21)
	MACE	High	None (13)	—	—	7 (14)	Not tested	Not tested
	MACF	High	None (24)	—	—	5 (21)	Not tested	Not tested
	MACG	High	None	—	—	71	Specific	86

	MAYB	High	29 (28)	Specific	45 (11)	76 (17)	Specific	100 (17)
(A.SW ♂ × A.CA ♀)F_1	MCSA	High	None (15)	—	—	30 (10)	Low	100 in A.CA (28) 20 in A.SW (40)
	MCSB	High	None (26)	—	—	None (21)	—	—
	MCSC	High	27 (15)	Not specific	29 (21)	20 (10)	Specific	79 (14)
			IN A.BY STRAIN			IN A.SW STRAIN		
(A.BY × A.SW)F_1	MWYA	Low	23 (13)	Not tested	Not tested	100 (19)	Not tested	95 (18)
	MWYB	High	None (17)	—	—	100 (13)	Not tested	Not tested
	MYSA	High	13 (15)	Not tested	Not tested	6 (16)	Not tested	Not tested
	MYSB	High	None (5)	—	—	50 (10)	Low	72 in A.SW (28) 19 in A.BY (16)
	MYSC	High	20 (10)	Not specific	88 in A.BY (17) 65 in A.SW (20)	6 (18)	Not specific	68 in A.SW (22) 82 in A.BY (11)

* Numbers in parentheses indicate numbers of mice tested.

eign genotypes (such as MSWB-SY and the variants of MCSA and MYSB), and one variant was specific but did not grow in preimmunized mice (DSWB). It is nevertheless most interesting that clear-cut variants with selective compatibility *could* be established from some tumors. Such variants were especially suitable for the serological and cytological analyses discussed in the following sections.

Six tumors that grew in some mice of *both* parental strains represent a particularly interesting group. From three sarcomas of this category (MACC, MACD, and MAYB), we selected variant lines specific for either one of the parental strains and refusing to grow in the opposite parental type or in foreign genotypes. The reciprocal variants derived from a given tumor were nevertheless not quite equivalent. Variants selected in the maternal strain grew in all maternal strain mice preimmunized against tissues containing the isoantigenic factors derived from the paternal strain. Variants selected in the paternal strain, as a rule, grew well in nonimmunized mice of the paternal strain and refused to grow in the maternal strain. Usually, much less than 100% of these variants grew in paternal strain mice preimmunized against tissues of the maternal strain. Thus a certain predilection for one of the parental strains was expressed even with tumors that gave rise to variants in both directions. To what extent the preference for the maternal strain is an essential feature of this behavior remains to be seen when information becomes available on tumors derived from reciprocal hybrids of the same genotype.

Two tumors (MSWC and MWYA) showed a considerable lack of specificity from the beginning, and grew more or less indiscriminately in both parental strains and in foreign genotypes. Extensive efforts to break up MSWC into sublines with specific compatibilities by consistent selection in preimmunized mice were entirely without success; all sublines maintained about the same degree of nonspecificity.

Considering now the various F_1 combinations for themselves, certain peculiarities appear in the different groups.

Sarcomas induced in (A ♂ × A.SW ♀)F_1 never gave rise to specific variants in strain A, in spite of rather extensive tests. With the A ♂ × A.CA ♀ combination, variants appeared in both directions but were more frequent in and, after selection, more compatible with the maternal A.CA strain than the paternal A type. This was the only group in which some tumors grew in less than 100% of the original F_1 type. Not much can be said of the (A ♂ × A.BY ♀)F_1 type since only two tumors have been tested; the variants obtained from one of them (MAYB) showed, nevertheless, a distinct preference for the maternal strain. Preference for the maternal type was also indicated by the tumors of the (A.SW ♂ × A.CA ♀) F_1 combination. The (A.SW × A.BY)F_1 sarcomas were finally characterized by an unusually high tendency to grow in the A.SW parent in three cases and an apparent nonspecificity of the selected variants tested serially.

Serological tests

In a previous work (E. Klein *et al.*, '57), the original line of the MSWB tumor [induced in an (A × A.SW)F_1] was compared serologically with its selected variants that grew in the A.SW parental strain in a high percentage and in a specific way. When inoculated into A.BY or A.CA mice, the original line provoked formation of isoantibodies reacting with specific isoantigenic factors of *both* parental strains. In contrast, variants induced only isoantibodies directed against components derived from A.SW. This was true for both hemagglutinins and soluble cytotoxic antibodies detected by their ability to kill normal lymphocytes. Also, the original line regularly absorbed anti-A antibodies from isoimmune sera but the variants consistently failed to do so. This led to the conclusion that the variants had lost the specific isoantigens determined by $H\text{-}2^A$ and differing from $H\text{-}2^S$ concurrently with development of their ability to grow regularly in the A.SW parental strain.

These findings were followed up in more detail by Bayreuther and Klein ('58). Here the isoantigens determined by the *H-2* locus were studied individually. The original line, MSWB, contained the isoantigenic components D and K (derived from the strain A parent), S and G (derived from A.SW), and E and F (common to A and A.SW). On the other hand, *both* D and K were regularly absent from the variants but S, G, E, and F were still present. Besides confirming the previous findings, this establishes definitely that at least two components were lost simultaneously from the variant cells.

After this was found true for the usual variants obtained in A.SW, we attempted selection of variants that had lost only one isoantigenic component. As suitable hosts for such experiments, (A.SW $\times$ DBA)F_1 and (A.SW $\times$ C3H)F_1 were chosen. As far as is known (Hoecker, '56), the isoantigenic composition of (A $\times$ A.SW)F_1, determined by *H-2* is CDEFKH/CEFGS; the corresponding situation for (A.SW $\times$ DBA)F_1 would be CEFGS/CDE^{d}FH; and for (A.SW $\times$ C3H)F_1, it would be CEFGS/CEHK. Upon inoculation of the MSWB tumor of the original line, A.SW $\times$ DBA mice would therefore respond with anti-K, and A.SW $\times$ C3H mice with anti-D antibodies. Variants arising in such hybrids might represent monofactorial losses, if specific for the hybrid in question. Until now, only one variant that may belong to this category has been obtained. It arose in one of three (A.SW $\times$ DBA)F_1 mice inoculated with the original line of MSWB. Upon further testing, it gave the following results: (A.SW $\times$ DBA)F_1, 11/11; (A.SW $\times$ DBA)F_1 preimmunized against the original line of the tumor, 2/2; (A.SW $\times$ C3H)F_1, 1/9; A.SW, 2/6; and A.SW preimmunized against original line, 0/3. Serological characterization and further transplantation tests on this line are in progress.

Serological tests have also been performed on tumors giving rise to variants in *both* parental strains. Data for MACC and its derived sublines MACC-A and MACC-C are shown in table 7. Although MACC elicits formation of isoantibodies

TABLE 7

Hemagglutinating power of serum from A.BY mice immunized with MACC and its variants

TUMOR LINE USED FOR IMMUNIZATION	SOURCE OF TISSUE USED FOR SERUM ABSORPTION	SOURCE OF RED CELLS	EFFECT OF THE FOLLOWING ANTISERUM DILUTIONS ON RED BLOOD CELLS [a]								CONTROL (NO ANTI-SERUM)
			4	8	16	32	64	128	256	512	
MACC [origin: (A × A.CA)F_1]	A.BY	A	+	++	++	++	++	++	+	+	—
		A.CA	++	++	++	+	+	+	—	—	—
	A	A	++	—	—	—	—	—	—	—	—
		A.CA	++	++	++	++	++	+	—	—	—
	A.CA	A	++	++	++	++	++	++	++	+	—
		A.CA	—	—	—	—	—	—	—	—	—
MACC-A (A variant)	A.BY	A	++	++	++	++	++	++	+	+	—
		A.CA	++	+	±	—	—	—	—	—	—
	A	A	±	±	±	—	—	—	—	—	—
		A.CA	—	—	—	—	—	—	—	—	—
	A.CA	A	++	++	++	++	++	++	++	+	—
		A.CA	—	—	—	—	—	—	—	—	—
MACC-C (A.CA variant)	A.BY	A	++	++	++	+	—	—	—	—	—
		A.CA	++	++	+	++	++	++	+	+	—
	A	A	—	—	—	—	—	—	—	—	—
		A.CA	++	++	++	++	+	+	+	+	—
	A.CA	A	+	±	±	±	—	—	—	—	—
		A.CA	—	—	—	—	—	—	—	—	—

[a] — No agglutination.
+ Clear positive agglutination.
++ Strong complete agglutination.
± Some agglutination.

capable of agglutinating both A and A.CA erythrocytes in a specific way, the variant MACC-A selected in the A parental strain failed to provoke hemagglutinins that would react with A.CA erythrocytes after the components common for A and A.CA had been absorbed with tissue of strain A origin. Conversely, the variant MACC-C, selectively compatible with the A.CA strain, could no longer provoke isoantibodies specifically reacting with red cells of strain A origin. Closely parallel results were obtained when cytotoxic isoantibodies were tested for their ability to kill normal lymphocytes (K. E. Hellström, unpublished), according to the method of Gorer and O'Gorman ('56). (Less-extensive tests were performed with tumors MACD, MYSB, MAYB, and some of their variants. The results were essentially similar and will not be reported here in detail.)

Two features are particularly noteworthy in connection with these findings. The factor $H\text{-}2^A$ (carried by A) differs from $H\text{-}2^F$ (strain A.CA) with regard to at least five isoantigenic products, according to Hoecker ('56); the known components are currently denoted as CDEFKH ($H\text{-}2^A$) and GHI ($H\text{-}2^F$). Thus a disappearance of the isoantigens specific for A, as happens in MACC-C, would mean the loss, or at least the very substantial decrease, of not less than five components. It may be pointed out that MACC-C grew in all A.CA mice preimmunized against tissue of strain A origin. On the other hand, variant MACC-A, which should have lost at least two components — according to the results of hemagglutination and cytotoxic tests, grew in only 70% of A mice preimmunized against A.CA tissue. The different transplantation behavior of the two variants was thus not reflected by the serological tests. The same was true for sarcoma MACD and its variants.

Variant formation in the MSWB sarcoma

The MSWB sarcoma was the first tumor encountered that exhibited a regular variant formation in one of the parental strains. Repeated testing of the original line resulted in

growing tumors in 15–25% of the A.SW mice but none in mice of the other parental strain (A). Tests in A.SW mice preimmunized against tissue of strain A origin decreased the yield of tumors to 2%. On the other hand, variants of MSWB grew in 92% of the A.SW mice and in 86% of the preimmunized A.SW mice.

The question arose whether formation of variant tumors at different times represents a series of independent events or repeated selection of just one or a few clones of variant cells. Further studies on this problem will be the subject of a separate publication (Bayreuther and Klein, '58). In this paper, only findings relevant to the present discussion will be briefly summarized.

The problem was approached by two different means: inoculation of small cell numbers and studies on the detailed chromosome cytology of the original tumor line and its different variants. The former approach was based on the argument that variant clones preexisting as a minority of the population (and they would have to be a rather small minority in view of the high selective advantage of compatible cells; see Klein and Klein, '56b) would usually be eliminated by the use of very small inocula. Unless new variant clones formed rapidly during the subsequent development of the small inoculum into a large tumor, the frequency of growing tumors in A.SW mice would have to decrease considerably with decreasing inoculum size. This was tested in two different ways: in one type of experiment, a series of tenfold dilutions containing 30 to 300,000 cells were inoculated into the original $(A \times A.SW)F_1$ genotype, and the resulting tumors were subsequently tested for variant formation in A.SW mice. There was no significant difference among the different groups; all gave rise to variants in 10–25% of the mice, and no correlation was apparent between inoculum size and variant formation. To avoid the interposition of one transfer in $(A \times A.SW)F_1$ mice and the consecutive increase in the number of cell generations that elapse before the actual testing,

a second series of experiments was performed in which nine different serially diluted cell suspensions containing 30 to 10 million cells were inoculated directly into A.SW mice. Compatible $(A \times A.SW)F_1$ were inoculated simultaneously as controls. All controls developed growing tumors down to an inoculum of 40 cells. In the nine experiments, an average of 26% of A.SW mice developed growing tumors. With the possible exception of one experiment, there was no apparent tendency for decreased variant formation with decreasing inocula. A typical experiment is shown in figure 1. Four groups containing 13 A.SW mice each were inoculated subcutaneously with 30 to 30,000 cells. Five $(A \times A.SW)F_1$ mice served as controls for each group. All controls developed growing tumors except two of the five mice that received the smallest inoculum. Ten days after inoculation, when the first palpation of the A.SW groups was made, there was an apparent correlation between tumor incidence and inoculum dose (fig. 1). This turned out to be spurious, however, since regression of some tumors in the group that received the largest inoculum and progressive appearance of new tumors in the groups originating from small inocula resulted in a converging tendency and a closely similar final incidence of progressively growing tumors in all groups, 46% on an average. This was a rather high frequency, and it may be pointed out that the incidence figures varied quite considerably from experiment to experiment but showed no obvious correlation with the dose of inoculum. In one experiment, there was nevertheless an apparent decrease of variant formation with de-

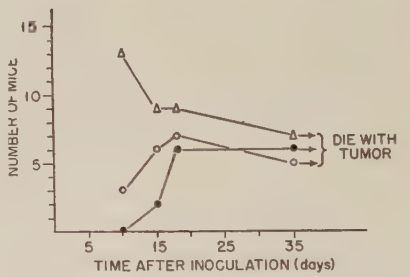

Fig. 1 Number of A.SW mice with palpable tumors after the inoculation of various numbers of cells of the MSWB sarcoma. The table shows the end results.

NO. OF CELLS	MICE KILLED BY TUMOR/MICE INOCULATED	PERCENTAGE KILLED
3×10^4(△)	7/13	54
3×10^3(○)	6/13	46
3×10^2(●)	7/13	54
3×10	4/13	31

creasing inoculum size (40,000 cells inoculated, 33% variants; 4000, 44%; 400, 16.5%; 40, 0%); but, since this was an isolated observation, its significance might be questioned. On the whole, these experiments, indirect as they are, suggest that the repeated variant formation by the MSWB tumor is not merely the consequence of repeated selection of the same clone or clones, existing as a permanent minority in the tumor-cell population, but of repeated independent events.

More-direct evidence to this effect was obtained by Bayreuther, who examined ten different variant lines with regard to their chromosome cytology (Bayreuther and Klein, '58). He found that the original line had a diploid modal chromosome number and contained one typical marker chromosome. During the early passages, there was a considerable spread of chromosome numbers around the modal value, but after serial transfer in the original $(A \times A.SW)F_1$ host, the distribution became much narrower. The modal type remained constant during 11 passages. All variants differed from the original line and, with one exception, also from one another with regard to their detailed chromosome cytology. This was expressed more as a difference in marker chromosomes than in chromosome number. Nine lines remained in the diploid or hyperdiploid range, although the distribution of their chromosome numbers showed clear individualities and differences from the original type. Eight of them contained the characteristic MSWB marker chromosome and five acquired one or more new, easily identifiable markers. One line changed its modal chromosome number to the hypertetraploid range. In view of Hauschka and Levan's findings ('53) regarding correlations between chromosome number and strain specificity in ascites tumors, it may be of interest to point out that this variant line (MSWB-SY in table 1) was the only one that showed a considerable tendency to grow in genotypes other than A.SW.

In one instance, two variants that arose from the *same* original inoculum in two different A.SW mice, possessed ex-

actly the same chromosome morphology, differing from the original line but mutually identical. In contrast to the other lines that must have arisen as a result of independent chromosomal rearrangements, these two variants most probably represent the repeated selection of one and the same clone.

Bayreuther has also examined some other tumor lines besides MSWB, mostly DSWB, MNSB, MSWC, MNSC, MNSD, and MACC. Each line had its individual chromosomal characteristics, and each stem line differed from both the normal idiogram and other tumor lines in chromosome number and detailed morphology. In this material, there was no obvious correlation between the chromosome number of the stem line and transplantation specificity, viz., the ability to give rise to variants. DSWB, MNSB, MNSC, and MACC were highly specific and near tetraploid; MSWC, the least specific among the tumors tested, was closest to diploid in the whole material since its stem-line cells contained 43 chromosomes.

DISCUSSION

The present study demonstrates some of the advantages and also some of the pitfalls inherent in the use of *histocompatibility* genes as markers for cytogenetic studies on neoplastic cells. The main advantages of the system are that genetically compatible cells possess an absolute selective advantage in a mixture with incompatible ones, and that compatibility can be made to depend, if not on the behavior of a single gene, at least on a single compound locus or a small chromosome segment. The pitfalls arise predominantly from nonspecific background growth of incompatible cells in some tumor-host combinations, leading to the development of false positives. It seems necessary to distinguish between such growth, occurring in the presence of and in spite of the homograft reaction (clearly selective in other situations) and of true variants that have become genetically compatible with their new host. The reason that certain tumors are nonspecific has not been found. The present discussion can be restricted to the specific

tumors, which represent the majority of our material (21/23). We might consider as true variants those that arose in a small proportion of mice belonging to one of the parental strains, grew in a high frequency when further tested in the same parental strain, including hosts preimmunized against tissue isoantigens of the opposite parental strain, and consistently refused to grow in the opposite parental strain or in other IR mice carrying foreign alleles at the *H-2* locus. It is thus necessary to establish for each variant whether it breeds true to type, resists previous immunization against the opposite parental type, and shows selective compatibility. Serological evidence is of considerable value in confirming the loss of detectable isoantigens derived from the noncorresponding parental strain.

Considering these criteria, a puzzling feature of the present study is that the ability to form variants in one or both parental strains seems to vary from tumor to tumor, in spite of the fact that the material used consists exclusively of sarcomas with the same general type of histology and induced by the same carcinogenic agent in the same tissue of coisogenic mice with an A/Sn background. Is it possible to interpret this finding as indicative of difference in the stability of the compound *H-2* locus or the *H-2*-bearing chromosome segment in the different tumors? Several factors enter into such a consideration, and it must be emphasized that it is not possible to distinguish critically between genes and gene products in somatic cells. Nevertheless, some findings provide rather suggestive indirect evidence. Growth of variants in preimmunized mice, their selective compatibility with their new hosts, and loss of serologically detectable isoantigens derived from the opposite parental strain are at least not incompatible with a genetic change. Since *several* isoantigenic factors are lost simultaneously and crossing over has now been demonstrated *within* the compound *H-2* locus (Gorer, '56), the change might be postulated to be more extensive than a single point mutation. Alterations involving a larger chromosome segment or an entire chromosome would be more plausible

and by no means incompatible with the cytological findings indicating that variant formation is accompanied by extensive chromosomal rearrangements. It might be postulated that these rearrangements, different in detectable detail for different parallel variants, involve the *H-2* region. Alternatively, it would be conceivable that the *H-2* region is not necessarily participating in the rearrangements in a direct way in all cases but its mutability may change in the new genetic background consequent to the rearrangements.

That certain tumors give rise to variants in the direction of *one* parental strain only is very puzzling. Whenever several tumors in the same genotype group showed this behavior, the favored parental strain was the same for all of them. A random genetic change might have been expected to proceed in both directions with about equal frequency. The fact that this does not happen with certain tumors might be attributable to some cytogenetic peculiarity involved in the system that could make one change more likely to occur than the other. It is also possible, however, that the peculiarity lies in the parental hosts rather than in the tumor cells. The isoimmune response provoked by different isoantigen combinations varies greatly (Hoecker, '56). The homograft reaction might be more rapid and more efficient in one parental strain than the other, owing to the differences in the strength of the different components at work. This would allow more cell generations to elapse before regression occurred in one parental strain than in the other. A larger number of cell generations would facilitate appearance and selection of viable variants. That this may be a relevant mechanism is indirectly indicated by the observation that, although tumor MSWB gave rise to 20–25% variants in the A.SW parental strain, the frequency of variants dropped to 2% in preimmunized A.SW mice, where the homograft reaction intervenes much earlier and more efficiently.

Differences in the probability that tumor cells will transgress the different isoantigenic barriers of the *H-2* system are

indicated by the distribution of completely specific tumors that refused to give rise to variant formation under the experimental conditions. Four out of seven such tumors belonged to a single genotype, A × A.SW. In contrast, there were no tumors among the five derived from A.SW × A.BY whose growth was entirely restricted to the original type; low specificity was common among the variants tested.

The existence of a favored parental strain of variant formation, characteristic for the genotype group, comes to expression not only in tumors that change in one direction only but also in those that produce variants in both parental strains. The reciprocal variants obtained from a given tumor were never quite equivalent in these cases, but one of them grew better in its corresponding preimmunized host than the other. The strain of preference was the same as indicated by those tumors of the same genotype that changed in one direction only. Thus for each genotype except A.SW × A.BY, one parental strain can be pointed out as the favored site of variant formation: (A♂ × A.SW♀)F_1, A.SW; (A♂ × A.CA♀)F_1, A.CA; (A♂ × A.BY♀)F_1, A.BY; (A.SW♂ × A.CA♀)F_1, A.CA; (A.BY × A.SW)F_1, A.SW (?; unclear). For the first four hybrid combinations, where the paternal and maternal strains were held constant and could be identified, the strain of predilection coincides with the maternal type. Unfortunately, the paternal strain is the same in three cases (A), and it would perhaps be premature to conclude that a maternal influence is regularly involved. Tumors induced in the reciprocal hybrids will have to be tested and the present tumors checked in selected F_2 or backcross mice equipped with the nucleus of the original maternal and the cytoplasm of the original paternal strain before a definite conclusion can be reached about this. Studies of this type are in progress. Meanwhile, results are at least suggestive of a maternal effect, which would indicate the presence in the tumor cells of isoantigenic factors derived from some nongenetic component of the mother. We would have to infer

that this component is being maintained during differentiation and tumor development. By acting as an isoantigen in the paternal strain, the component would prevent the manifestation of genetic variants. The assumption that variants toward the paternal strain do occur is confirmed by such tumors as MACC and MACD. Serological tests on such variants indicate that they have lost the *H-2*-determined isoantigenic components derived from the maternal strain, at least to the extent that these are no longer detectable by the available tests. In spite of this and in contrast to the maternal variants, they do not grow in more than a fraction of paternal strain mice preimmunized against tissues of the maternal strain. Thus some factors derived from the maternal strain and having isoantigenic activity but unable to elicit hemagglutinin production or the formation of humoral cytotoxins detectable by conventional methods must still be present in these cells. We might speculate that this would be some cytoplasmic, self-reproducing component. Since this would have far-reaching implications, involving both the mechanism of differentiation and that of carcinogenesis, more-critical tests are yet needed to establish its existence. A possibly analogous case was found by Hauschka *et al.* ('56) in backcross tests on a lymphoma of strain DBA origin. These workers interpreted their findings as an enhanced histocompatibility, associated with the presence of the X chromosome derived from the original DBA strain. It is not possible to distinguish between X-chromosomal and cytoplasmic factors in their test system, however. In our system of coisogenic mice, the role of the X chromosome can be excluded, and the evidence points toward the possible significance of some cytoplasmic or other maternal factor.

Finally, some consideration should be given to the possible significance of these results with regard to the mechanism of carcinogenesis by methylcholanthrene. In similarity with the results of Ford *et al.* ('57) on reticular neoplasms of the mouse, the present results indicate the existence of a cyto-

genetic individuality in different tumors of closely parallel origin. In our work, this individuality is indicated by the differences in biological behavior related to the *H-2*-bearing chromosome segment and also by the chromosomal individuality and uniqueness of each tumor line. Since the characteristics of each line seem to be stable and to breed true during serial transfer, dismissing these differences as mere secondary consequences of the same basic neoplastic transformation would not seem very appropriate. As we have suggested previously ('58), it would probably be more conservative to assume that rearrangements of chromosomal material can play an important role in some cases of neoplastic transformation. But these rearrangements do not have to be the only cellular mechanisms that can lead to the composite and heterogeneous group of end results that we perceive as neoplasia. Also, a neoplastic cell might be produced as a result of various kinds of chromosome rearrangements. This would not be very surprising; all that has to change in a cell for it to become neoplastic is its competence to respond to the normal mechanisms that maintain homeostasis, whatever their exact nature might be. We do not know how this competence is brought about, but we have every reason to believe that the underlying cellular organization is very complex and probably involves the participation of a multitude of both nuclear and cytoplasmic factors. If so, it would be only natural if the normal organization could be upset in many different ways and if the cellular mechanism underlying the development of malignancy could proceed along a multitude of different pathways.

SUMMARY

Somatic cell variation seems to be particularly relevant to the understanding of the development and progressive evolution of tumor-cell populations. For studies of the occurrence and nature of somatic variation *in vivo,* suitable and specific marker genes are essential. Selective systems that permit detection of specific gene products in single cells and their clonal derivatives must also be worked out.

Histocompatibility genes of the mouse seem to represent the only available system that can meet these requirements. By the use of Snell's IR mouse lines, genetic variation may be restricted to one small chromosome segment. F_1 mice produced by a cross between two IR lines and differing only with regard to the allelic substitution at the *H-2* locus seem to be particularly suitable for studies of somatic cells. Allelic differences at this locus constitute an exceptionally strong barrier against homotransplantation. Normal or neoplastic tissues of such F_1 mice are usually transplantable only to genetically identical hybrid hosts but not to any of the parental strains, where they provoke a homograft reaction directed against isoantigens determined by the *H-2* allele derived from the opposite parental strain. Mutation of one of the *H-2* loci in a heterozygous tumor cell may convey a specific compatibility on its bearer; however, when tested in one of the parental strains, it would thereby become detectable. These considerations led to the following experiments.

1. Sarcomas were induced by methylcholanthrene in four IR lines with an A/Sn genetic background and their various F_1 combinations. Model experiments involving the artificial mixture of known numbers of tumor cells derived from the parental strains and the F_1 mice demonstrated that even a very small randomly admixed compatible cell fraction has an absolute selective advantage in its corresponding host. This is true in spite of the complete destruction of the incompatible majority of the population by the homograft reaction, even where compatibility is a matter of a single gene difference. In fact, the homograft reaction directed against the incompatible majority stimulates the growth of the compatible minority (Klein and Klein, '56b).

2. A series of sarcomas was induced by the same dose of methylcholanthrene in the same tissue of various F_1 combinations of the four coisogenic-resistant lines. Tumors were tested by transplantation to the parental strains and other known genotypes. Seven tumors grew exclusively in their original F_1 host genotype and did not give rise to variant

sublines in any of the parental strains under the conditions of testing. Eight tumors gave rise to a certain proportion of variants capable of growth in *one* of the parental strains. In most cases, variants were specific for the parental strain of selection upon subsequent testing. No variants were obtained in the other parental strain with these tumors. For each F_1 genotype, one parental strain was clearly preferred to the other as the favored site of variant formation. Six tumors tested gave variants in both parental strains. A predilection for one of the parental strains, characteristic for the F_1 combination used, was clearly expressed even in this case. Two tumors were nonspecific and grew in both parental strains and also in foreign genotypes. It was not possible to select variant lines with selective compatibility from these strains.

3. Some tumors and their selected variants were tested by serological methods, involving both hemagglutination and cytotoxic tests, for the presence of certain isoantigens determined by the *H-2* system. The findings were closely parallel to the transplantation tests; they revealed a specific loss of detectable isoantigens concomitantly with development of the ability to grow in one parental strain.

4. Detailed cytological studies performed on a representative collection of F_1 tumors by Dr. Klaus Bayreuther showed that each tumor was characterized by a definite stem line different from the normal idiogram and from other tumor lines. There was no obvious correlation between chromosome number and strain specificity or the ability to give rise to variants.

5. One tumor (MSWB) that regularly gave rise to a certain proportion of variants in one parental strain was examined in detail. The cytological evidence of Bayreuther indicates that the occurrence of variants at different times represents mostly independent events. This was also confirmed by experiments involving the inoculation of very small numbers of cells. As a rule, the variants differ from both the original line and each other with regard to their chromo-

some cytology. Several lines have individually characteristic marker chromosomes.

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OPEN DISCUSSION

MITCHISON [1]: I would like to add two points to what you have said, Dr. Klein. First, the coisogenic strains still differ at other loci in addition to the *H-2* locus. The additional differences may be more important for some types of tumor than for others. When leukemias are placed in the same situation as your sarcomas, the additional differences seem to play a part in determining the outcome of transplantation. For example, the variant sublines from an initially heterozygous leukemia behave differently in normal and preimmunized mice of the parental strains. This may be because preimmunization increases the effect of secondary antigenic differences other than *H-2*.

The second point concerns tumors transplanted into the parental strains with no takes. I have a case of a heterozygous leukemia that behaves in this way. But the tumor can still be modified by passage by the Koprowski method through suckling mice of one of the parent strains and will then grow in adult mice of the parent strain. The change is permanent, for the variant can be maintained by passage in the F_1. Interestingly enough, this one tumor also shows a preference for one of the parent strains.

[1] N. A. Mitchison, University of Edinburgh.

HAUSCHKA[2]: In working with the histocompatibility genetics of ascites tumors, one often finds unusually close experimental agreements with Mendelian expectation. Such data establish faith in the relative uniformity of the antigenic types of certain neoplastic cell populations and in the feasibility of Dr. Klein's experimental scheme for recognizing rare antigenic mutants.

For example, the DBA/2 mouse lymphoma that we tested in resistant backcross (RBC) and (DBA/2 $\times$ C3H)F_2 gives take percentages indicating that the tumor differs from the resistant C3H parent stock by two H factors: 46 takes (progressive growth causing death of the host) were obtained in 200 RBC mice injected intraperitoneally; expectation on a two-gene basis is 50 takes, or 25%. There were 114 takes in 207 F_2 mice, Mendelian expectation being 116, or 56.2%.

This almost embarrassing agreement of experimental yield with theoretical anticipation becomes less close when we scrutinize the data for the 200 RBC mice by considering separately the takes in 100 females (35%) and 100 males (11%). The convincing two-gene result applies only to the sum of both sexes, compatibility of the females with the transplantable lymphoma being considerably greater than the 25% expectation and that of the males considerably less.

Among other host factors that can influence compatibility in these tests are an X-linked modifier, a milk influence, age of the host, and transplantation site. Depending on the deliberate combinations of these take-promoting or -inhibiting factors operating together in different backcross experiments, take percentage of the DBA/2 lymphoma in essentially the same RBC hybrid genotype may range all the way from about 5% (suggesting four genes) to an upper ceiling of 50% (suggesting one gene). This ceiling is firmly set by the strong antigenic *H-2* difference between D in the tumor and K in the refractory hosts.

[2] T. S. Hauschka, Roswell Park Memorial Institute.

To our knowledge, there are only two chromosomal happenings that could significantly expand the upper 50% limit determined by the *H-2* difference in this test system: either a somatic mutation at D, such as Dr. Klein has observed, or isoantigenic weakening attributable to gross nuclear changes in the graft cells from diploid to heterploid.

KLEIN: I certainly agree with Dr. Hauschka that the results of F_2 and backcross tests between two randomly chosen inbred strains can be modified by many different host factors. What we should like to concentrate on, however, is the behavior of the *H-2*-determined isoantigenic differences between host and tumor cells. We can concentrate on these and eliminate much of the irrevelant host influences by using IR lines differing at the *H-2* locus rather than randomly chosen inbred strains that differ at many other *H* loci. Some of these other differences are quite weak as transplantation barriers, and it will depend on various environmental factors whether or not they will come to expression. *H-2* is a strong barrier and can be made even stronger by preimmunization. Tests in preimmunized IR mice differing at the *H-2* locus are therefore less likely to be influenced by variations of the host environment than conventional F_2 of backcross tests.

OWEN [3]: I feel impelled to submit a comment that does not reflect at all on the beautiful work that Dr. Klein has done, but that I think may suggest some reservation in interpreting the genetic changes he has observed. This reservation relates, in the field of immunogenetics, to the significance of symbols like C, D, E, F, G, and H. These are described as antigens in a complex, but in many instances they may reflect not necessarily a set of separable structures in a complex antigen, but simply fractions of the heterogeneous antibody populations in immune sera, cross reactive in different patterns with sets of related antigens. These antibody populations can be cut in different ways, depending on how many related antigens are available to fractionate and test them. I raise this point, of course, with reference to the nature of the change

[3] R. D. Owen, Oak Ridge National Laboratory.

that needs to occur in order to produce, say, a change in two symbols in an "antigenic formula," and, as I think Dr. Mitchison was implying, to suggest that when one seems to observe positive changes in a block of symbols that would suggest an addition of some sort, he may be observing only a single, simple change to a related antigen showing a new pattern of cross reaction with the reagents at hand.

SNELL[4]: On the question of recombination at the *H-2* locus, I certainly agree with Dr. Owen that we cannot close our minds to more than one possibility. I ought not to be talking about this because Dr. Gorer has done most of this work. Something that either is recombination or mimics it has been found in three laboratories. Dr. Gorer has gotten several cases in which, from F_1 mice heterozygous at the *H-2* locus, new combinations of the different *H-2* antigenic factors, D, K, etc., have been obtained. He has found this several times, Mrs. Sally Allen found it in Chicago, and Dr. Hoecker has told me at this meeting that he has had it more than once. These recombinants appear with a frequency of about 1%.

As Dr. Owen said, we do not have markers on both sides of the *H-2* locus, and that is important. Until we do have such markers, I do not think we can be entirely sure of the meaning of these results. Mary Mitchell and other geneticists have recently demonstrated a new phenomenon, distinct from crossing over and called (among other things) "genic conversion," in which gene replication during meiosis leads to a new gene with attributes derived, not from one only, but from both members of a heterozygous gene pair. The new *H-2* types, which are produced with a frequency of approximately 1% by *H-2* heterzygotes, could be a result of this phenomenon. Although present evidence does not exclude this possibility, I personally think that it is unlikely.

FORD[5]: My approach to these problems is always very naive. When I first heard of Dr. Klein's results, knowing that Dr. Mitchison had had similar results with lymphomas with

[4] G. D. Snell, Jackson Memorial Laboratory, Bar Harbor.

[5] C. E. Ford, Radiobiological Research Unit, AERE. Harwell.

regard to the one-way takes (that is to say, a take in one of the two parental strains in which the tumors arose), I naturally thought of how this could be explained. I also thought of my own work, which was concerned very largely with chromosomal changes.

The first possibility seemed to be one of duplication of the *H-2* locus on the one side, which would reduce the frequency with which the expected mutation occurs roughly as the square of the ordinary mutation rate. That would provide a specificity of change characteristic of the tumor. The other possibility that occurred to me, the *H-2* locus being a complex one, was that for some of these mutational changes we would need two separate steps so that we would have a similarly reduced frequency as the square approximately. That would be the specificity of the allele, particularly of the genotypic combination used. It seemed to me that really neither of those two explanations applied to Dr. Klein's data.

Klein: I think that no explanation can be excluded at the present stage. I believe that the three major explanations for the unidirectional preference of the change can be listed as follows: (1) some cytogenetic peculiarity of the tumor cells; (2) some property inherent to the different host-tumor combinations, such as differences in reactivity against different isoantigenic barriers; and (3) a maternal effect of some kind.

Until there is evidence available in tumors induced in the reciprocal hybrid types, it is not possible to distinguish critically between these alternatives.

Hoecker[6]: I agree with Dr. Owen's reservations, but since I have been named in connection with the crossing over at the *H-2* chromosome region of the mouse, I might as well add the following information on work done in collaboration with Dr. Pizarro and Miss A. Ramos.

We tested recombinations between the D and K antigens and found a 1.4% value. This result fits very well with figures obtained by Dr. Gorer and Mrs. Allen. Since we had only

[6] Gustavo Hoecker, University of Chile.

one other marker gene, T, we cannot say what the gene order is. We also tried to see whether these antigens (the D, K, E combination present in A/Sn mice) appeared at different times in development. This would indicate two different loci. To our surprise, we found that they all appeared at about the same time. The suggestion therefore arises that the *H-2* chromosome region works as a physiological unit. However, since the two complementary combinations, D positive–K negative and D negative–K positive, were observed both by Gorer and us, this seems to be a good indication that we are dealing with, at least, two discrete gene entities.

RAPPAPORT [7]: Is there any difference if you do the reciprocal cross and then transplant from the F_1 hybrid to the mother versus the father?

KLEIN: This is what we are presently doing. We have no results yet.

LEDERBERG [8]: You described the selection of A or S homozygotes when A/S hybrid cells were inoculated into the parental lines. Could you also test for a specific host influence (e.g., transduction from the host to the tumor cells) by using an A/Y heterozygote as the screening host and then determining whether the types that grow in that host are A homozygotes or A/Y heterozygotes?

KLEIN: We have done that quite extensively. I did not include in the table all the types in which we tested tumors, but we tested them in all types, and occasionally obtained takes. For instance, an $(A \times A.SW)F_1$ tumor would sometimes take in $(A \times A.BY)F_1$ or $(A \times A.CA)F_1$, but so far as we have been able to ascertain, all these takes have been false positives; they did not breed true on repeated passage.

AUERBACH [9]: Can you do the experiment in such a way as to distinguish whether this change occurs in the host in which you screen or whether it has occurred before this in the F_1

[7] Irving Rappaport, University of California.

[8] Joshua Lederberg, University of Wisconsin.

[9] Charlotte Auerbach, Oak Ridge National Laboratory; on leave from University of Edinburgh.

mouse. If you use as a host a preimmunized mouse, the mutation has presumably occurred before. Have you ever tried to see whether there is an increase in the percentage of takes in preimmunized mice if you allow a longer time or several generations of transplants in F_1 mice in which there would not yet be any selection?

KLEIN: I am afraid we have not done that. We usually do not transfer these tumors for any lengths of time at all, but test them, if possible, all through the first generation. We freeze a large series of samples and thaw them out one by one and test them. The only evidence we have that may be relevant to your question is that this particular tumor, which preferentially changes to the A.SW, gives 20–25% variants in nonimmunized mice and only about 2% variants in immunized mice. It is possible, of course, that this 2% represents the preformed variants although this is not certain. In one particular case, however, where we had two variants arising in two different individual A.SW mice from the same original inoculum pool, Bayreuther found them to have exactly the same chromosomal constitution. I think here we have very suggestive evidence that we were dealing with the repeated selection of the same preformed clone. This is the only evidence we have at the moment.

BRAUN [10]: Do you have any observations, or suggestions, to explain the increased incidence of takes when you transplant a small number of compatible cells in conjunction with a large number of noncompatible cells, compared with the lesser incidence of takes when you transplant only a small number of compatible cells? Is this merely a matter of cell mass or is something else involved?

KLEIN: My colleague, Dr. Révész, is particularly studying this question. He finds that, by mixing a very small number of viable cells that are compatible with their host with a very large number of cells that are irreversibly inactivated by X rays, he gets very much the same effect. Also, if he

[10] Werner Braun, Rutgers University.

adds viable isologous liver cells, they will enhance the growth of a small tumor inoculum. However, if he adds tumor cells inactivated by heat or just by prolonged incubation at 37°C., he does not get any stimulation whatsoever.

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MUTABLE LOCI AND DEVELOPMENT OF THE ORGANISM [1]

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ONE FIGURE

It is a widely accepted genetic doctrine that each nucleus in an organism receives the entire complex of genes. According to this view the diverse cell lineages arising during ontogeny do not reflect qualitative differences at the gene level. Unstable loci, however, provide an obvious exception to this rule. Genetic heterogeneity within the individual arises as a result of frequent somatic mutations at these loci. Is the nuclear diversity originating in this way significant for ontogenetic differentiation? When genetics is looked at from the standpoint of a theory of the organism, do mutable loci provide an escape from the rigors of the discipline relative to development? This interesting possibility has been proposed by McClintock ('51, '56a,b), whose controller hypothesis, although mainly concerned with mutation, embodies the idea that mutable loci represent a mechanism of ontogenetic differentiation.

The ultimate control of development resides in the genes. Development involves differentiation, a sequence of specific events, ordered in time and space, whereby the structure and physiological properties of particular groups of cells become more or less fixed. Since this specialization of structure and function of cell groups is mediated by determinative processes resting on gene action, it is necessary to suppose that gene activity is differential during ontogeny. One set of gene-con-

[1] Paper No. 686 from the Department of Genetics, College of Agriculture, University of Wisconsin.

trolled processes is promoted under one set of circumstances and different ones under other conditions, the order of the several events, in general, being invariable. The problem of development in these terms is one of the basis of unified differential activation of the genes. The particular question to be considered in this article is whether mutable alleles provide clues to the process whereby gene potentialities are thus translated into determinative action.

VARIEGATED PERICARP IN MAIZE

During recent years my students and I have been studying a highly mutable character, variegated pericarp in maize. The subsequent discussion will relate largely to the results of this work because I am familiar with it at first hand and it has provided significant evidence on the particular aspect of mutable alleles now at issue.

The variegated pericarp allele has been shown to be a dual structure (Brink and Nilan, '52). One component is considered to be the gene for self-red pericarp, P^{rr}, located near the mid-region of the short arm of chromosome 1. The other has been termed *Modulator* (*Mp*). The P^{rr} gene, when resident alone at the *P* locus, is entirely conventional in genetic behavior. Emerson ('17) and Anderson ('24) have shown it to be the top dominant in a stable series of multiple alleles conditioning pericarp and cob color.

Interest in the present connection focuses on *Mp*. The relation of this element to variegated pericarp may be summarized as follows:

1. *Modulator,* when present at the *P* locus in conjunction with the P^{rr} gene, suppresses the pigment-producing action of the latter. For convenience, we may speak of the $\overline{P^{rr}Mp}$ combination as a *P* allele, conditioning colorless pericarp and cob in the absence of mutation.

2. This colorless allele is unstable and mutates to red frequently and irregularly in somatic tissue. The sporadically occurring mutations from colorless to red in the developing

ear shoot of a $\overline{P^{rr}Mp}$ plant heterozygous for a stable colorless allele result in the collective phenotype known as medium variegated pericarp. Progeny tests show that such a variegated ear is a mosaic of genetically unstable colorless and genetically stable red tissue.

3. Mutation from colorless to red involves an extraordinary type of change, the nature of which McClintock ('51) was the first to discern in her classical study of *Dissociation* (*Ds*) and *Activator* (*Ac*). *Ac* and *Ds* were found to be transposable from one position in the genome to another. Brink and Nilan ('52) showed that, in the variegated pericarp, the change from colorless to red involved transposition of *Mp* away from the *P* locus, thus permitting normal expression of the pigment-producing action of the P^{rr} gene.

4. We found also, as McClintock ('51) had previously observed in *Ds,* that after removal from the *P* locus, *Mp* is not necessarily lost to the genome but often is implanted, as it were, at some other site.

5. The effect of a transposed *Mp* in a plant carrying a variegated allele at the *P* locus ($\overline{P^{rr}Mp} + Tr - Mp$) is to reduce markedly the frequency of colorless to red mutations, resulting in a phenotype called light variegated (Brink and Nilan, '52). Brink ('54) found that two transposed *Modulators* tend further to stabilize the variegated allele ($\overline{P^{rr}Mp}$) giving a phenotype referred to as very light variegated ($\overline{P^{rr}Mp} + Tr - Mp + Tr - Mp$). Mutations to red and to light variegated are complementary and frequently are found as twin spots on a medium variegated ear. Such a twin spot results from a mitosis that is differential at the *P* locus, the *Mp* element that is lost from one daughter nucleus (red) as a result of transposition away from P^{rr} becoming the transposed *Mp* in the other (light variegated) daughter nucleus. (Twinning, however, is sometimes more complicated than this.) Most light variegated mutant areas on medium variegated ears are too small to be detected directly, but progeny tests

show that they occur with a frequency only slightly lower than that of red stripes.

6. The last point to be noted here is that *Mp* and the unit that McClintock ('51) described as *Ac,* although discovered independently in unrelated strains, are the same, or similar, elements. Evidence for this conclusion is derived from three sources. Barclay and Brink ('54) found that variegated only, among the various common *P* alleles, promoted the *Ds* type of chromosome breakage, the specific test that McClintock ('53) developed for presence in the genome of *Ac.* Second, Fradkin and Brink ('56) observed that McClintock's *Ac,* when introduced into variegated pericarp plants, changed the phenotype from medium to light variegated. That is, *Ac* gave the same kind of effect on expression of the variegated pericarp allele as a transposed *Mp.* Finally, McClintock ('56b) has shown that *Ac* inserted at the *bronze* (*bz*) locus renders *bz* unstable. A variety of responses of *bz* to *Ac* have been observed. Certain of the effects resemble those of *Mp* at the *P* locus.

Variegated pericarp is of considerable antiquity in maize, so that *Modulators* derived from this allele by transposition may be expected to be encountered from time to time in nonvariegated pericarp strains. Conceivably *Ac,* which was found by McClintock in a colorless pericarp stock, originated in this way.

THE CONTROLLER HYPOTHESIS

McClintock ('51, '56a) has attempted provisionally to relate the evidence from mutable loci to the problem of ontogenetic differentiation. I shall summarize my understanding of her views on controllers as a background against which the evidence from variegated pericarp bearing on development may be considered.

1. The chromosome comprises two primary classes of self-replicating elements, genes and controllers. "Gene" and "controller" in this context are terms of convenience. They are used in the conventional way for coding results of progeny tests. No implications are intended concerning size, discrete-

ness, elemental composition, or organization at a higher level of the two kinds of factors.

2. The gene embodies a certain action potential for the locus. Controlling elements at or near the locus (and sometimes elsewhere in the genome also) directly determine the manner in which the potential inherent in the gene may be expressed during development. Phenotypic effects are regularly the result of interaction of genes and controllers; neither operates alone. Thus both kinds of elements are assumed to pervade the genome.

3. Controlling elements fall into systems the components of which function in an integrated way during ontogeny. The term "system" as used in this connection apparently implies nothing more than that a controller has a measure of specificity and that its action may vary according to the presence or absence in the genome of certain other controllers.

4. Genes and controllers may be noncomplementary in the sense that a given controlling element or system of controllers may regulate the phenotypic expression of more than one kind of gene. Similarly, a given gene may at different times be subject to regulation by different controller systems.

5. Controllers are subject to changes in state in somatic cells. A change in state may occur in response to the action of another controller.

6. Controllers influence development of the individual in precise ways by effecting within the nucleus "an orderly sequence of triggering responses" at the several gene loci.

7. Certain controllers may shift from one chromosome position to another without change in state. Such transpositions constitute one aspect of controller action in development.

8. Variegation is the result of a disarrangement within a controller system such that one or more controllers is out of phase, so to speak, with the other controllers or with the genes affected.

The controller hypothesis is both an interpretation of mutable loci and an attempt to make the mechanism of

differentiation more intelligible. It is important for heuristic purposes that we recognize at the outset the quite different relation of the concept to the problems in these two areas.

The theory is based on evidence from unstable loci, and its applicability to this phenomenon is directly testable. Various maize investigators, including my associates and me at Wisconsin, can attest to its great usefulness in this area.

Projection of the controller concept into the field of developmental physiology, on the other hand, is a far-ranging extrapolation, the justification for which requires close examination. Because I consider this question still open, I propose to discuss the results on variegated pericarp, more or less on a case basis, in relation to it. A few general remarks, however, are first in order.

All attempts to generalize concerning development have been speculative. It must be conceded, for example, that the widely held idea that genes comprise a qualitatively unchanging complex throughout ontogeny is more a logical construct than an experimentally proved fact. The field, therefore, is open for new hypotheses, even for one involving the contrary assumption that constitutive changes at the gene level are primary factors in differentiation.

The second general point concerns the criticism sometimes heard that, whatever the genetic basis of mutable loci, these loci are too restricted in distribution among various classes of organisms to serve as a point of departure in constructing a general theory either of mutation or development. The facts are too few and disparate to answer this question convincingly, but there is considerable evidence suggesting that mutable loci, as known in maize, for example, have counterparts in phenomena that have been studied from other points of view in a wide variety of organisms.

Unstable loci are common, of course, among cultivated species of flowering plants, in which variegated forms have been selected for their novelty value. The cases in *Drosophila virilis* that Demerec ('41) analyzed appear to be closely paral-

lel. Moreover, it is possible that the euchromatic-heterochromatic transpositions in *D. melanogaster* first reported by Muller ('30) and studied subsequently by Gowen and Gay ('34), Schultz ('36), Demerec ('40), and others reflect the same, or a similar, phenomenon. Cooper's significant observation ('56) that the wild-type eye in *D. melanogaster* becomes mottled in genomes carrying two extra Y chromosomes seems to be related. Variegation of the kind in question has been observed in ferns (Andersson-Kottö, '30). Newcombe ('53), Treffers *et al.* ('54), and Ishikawa ('57) have described what appears to be its counterpart in fungi. A classical case among bacteria is phase variation in *Salmonella* (Andrewes, '22). It seems not unlikely that these various phenomena will prove eventually to be basically alike.

Another argument aimed at discounting the general significance of mutable loci is that the latter represent a special class within the total complement. Rhoades' observation ('41) that the normally stable a_1 locus in maize was mutable in the presence in the same nucleus of another factor, *Dotted* (*Dt*), undermined this view. McClintock ('51) has since described other cases of the same sort. Of even greater significance was McClintock's discovery ('51) in maize of transposability from one chromosome to another of elements contributing to instability of gene expression. This is a mechanism whereby, presumably, any locus in a nucleus can be changed from a stable to an unstable condition, or *vice versa.* It is now clear that, in maize at least, the potentiality for instability pervades the genome.

SOMATIC SEGREGATION

The first question that may be asked is whether, during the course of development, variegated pericarp does in fact effect a form of somatic segregation by virtue of changes involving *Mp*.

The self-red and light variegated twin spots found on medium variegated pericarp ears provide a particularly clean-

cut positive answer to this question. In the simplest type of such twin spots, but not invariably, progeny tests show that *Mp* is no longer present in the nuclei of the red component, either at the *P* locus or elsewhere. Analysis shows that the light variegated cotwin, on the other hand, still possesses a variegated allele at the *P* locus and also carries an *Mp* unit elsewhere in the genome (Brink, unpublished). This transposed *Mp* sharply reduces the frequency of variegated to self-red mutations at the *P* locus, and so results in the light variegated phenotype. As pointed out earlier, this kind of twin spot can be accounted for in terms of a differential mitosis in a medium variegated cell heterozygous for a stable colorless allele whereby the *Mp* lost from the *P* locus in the case of the red daughter cell is acquired by the light variegated daughter cell as a transposed *Mp*. Thus the result of such a differential mitosis is production of two daughter cells genetically different from each other and from the parent cell and giving rise to different lineages.

MODULATOR AS A UNITARY GENETIC ELEMENT

Is *Mp* a unitary component of the germinal substance or does it vary continuously, as would be expected if it were amorphous and readily subdivisible?

Two lines of evidence point to the conclusion that *Mp* is a definite entity, the integrity of which tends to be maintained in the chromosomes. These relate to stepwise mutations of *Mp* and transposition from one position in the genome to another, frequently without change.

Data in support of the conclusion that *Mp* varies in stepwise fashion rather than continuously is afforded by Valentine's study ('57) of orange variegated in our laboratory. Orange variegated occurs as a relatively uncommon mutant from medium variegated, the rate being about one per 1000 gametes (Brawn, '56). This is about 1% as often as mutations of variegated to self-red in the same strain. The background on an orange variegated kernel is a rich reddish brown,

whereas the background in medium variegated is colorless, or nearly so.

The mutation from medium variegated to orange variegated has occurred independently eight times in our hand-pollinated cultures. Four of these mutations arose in one strain, two in an unrelated line, and one each in two other stocks. All eight give similar phenotypes. The orange variegated mutants promote the *Ds* type of chromosome breakage. They mutate to self-red pericarp by transposition of *Mp* away from the *P* locus, a complementary mutant class, light orange variegated, often arising in the process.

Valentine ('57) established that two mutations to orange variegated that occurred independently in a given inbred strain mutated with comparable frequencies to self-red pericarp. The observed values in a large-scale test were 53.4 and 47.9 mutations to self-red per 1000 kernels. The difference, 5.5 per 1000 kernels, is well within sampling limits. The parent medium variegated allele in this strain gave 86.3 mutations to red per 1000 kernels. Thus orange variegated shows approximately 40% reduction in rate of mutation to self-red as compared with medium variegated. The resulting self-reds in the two cases are phenotypically indistinguishable. The change in mutation rate to red suggests that the difference between medium and orange variegated is in the *Mp*, rather than the P^{rr}, component of the variegated allele.

Valentine's evidence is compatible with the view that the change from medium variegated to orange variegated is the result of a distinct stepwise mutation of *Mp*. The mutant *Mp*, as compared with the *Mp* in medium variegated, suppresses the action of the gene for red pericarp at the *P* locus less completely, allowing production of some pigment in most of the cells of the pericarp. Also it is transposed from the *P* locus about 40% less often. The recurrence of orange variegated as a distinctive mutant phenotype suggests that the number of mutant forms of *Mp* is not large.

The second body of evidence showing that *Mp* is a unitary germinal component is derived from a study by Kedharnath and Brink ('58). The plan of the experiment was to isolate a series of independently transposed *Modulators* from a given variegated pericarp allele within an inbred line and then to test whether the transposed *Modulators* were alike. The criterion for identity was the modifying action of the several transposed *Modulators* on the expression of a common variegated pericarp allele on a uniform genetic background. It will be recalled that a transposed *Mp* in a plant carrying a variegated allele at the *P* locus gives the light variegated phenotype. In essence, our test consisted in determining whether, with a given variegated allele at the *P* locus, introduction into the genotype of each of several transposed *Modulators* regularly gave the same light variegated phenotype.

Five independently occurring transposed *Modulators* within the W23 inbred line were assayed in this way. All five light variegateds thus produced gave the same frequencies of mutations from variegated to self-red, within sampling limits.

Eight transposed *Modulators* from another inbred variegated line, 4Co63, were similarly tested. With reference to their effect on mutations of the variegated allele to self-red, four gave a mean of 31.26 mutations to self-red per 1000 kernels and the others gave a mean value of 36.14. Transpositions within groups did not differ significantly, but the difference between group means was highly significant. Moreover, the "t" test showed that the difference (2.64) between the highest value in the lower group (32.51) and the lowest value in the higher group (35.15) exceeded the critical value (2.59), and hence was probably real.

Each of these eight transpositions was the result of a separate event. Evidence from other experiments suggests that the site to which *Mp* moved from the *P* locus was different in each case. If this is true, then one would not expect that distribution of the eight transposed *Modulators* into two

groups only was a result of location (position effect) in the genome. The more probable explanation is that two slightly different forms of *Mp* are represented. It would appear that, if the *Mp* in one of the 4Co63 groups is assumed to be standard, then a preference is shown in mutation for one other form. Whether the mutations occurred prior to or in the process of transposition is not known.

If the data from the W23 and 4Co63 strains are considered together, it is apparent that the integrity of *Mp* tends to be maintained in transposition, although changes may occur. Certainly there is nothing in these findings that excludes the view that *Mp* is a chromosome segment of limited size, comparable in this respect perhaps to a gene in the conventional sense but differing from the ordinary gene in being transposable.

The evidence just reviewed concerning distribution of mutable loci among different organisms, pervasive action of controlling elements in the maize genome, somatic segregation, and *Mp* as a unitary genetic element is compatible with McClintock's concept of the controller as a regulatory factor in development. The next data to be considered are difficult or impossible to reconcile with this hypothesis.

DISPENSABILITY OF *MODULATOR*

All the chromosome deficiencies known in maize are lethal in homozygotes. Most of them, in fact, are not transmitted through the male gametophyte, or only with reduced frequency. McClintock ('44) described some exceptional cases, involving minute portions of the short arm of chromosome 9, that passed through both pollen and eggs in Mendelian proportions. The resulting homozygous deficient plants, however, died as seedlings. These facts suggest that the entire maize genome is essential to viability. The question may be asked whether *Mp* also is indispensable to the maize plant.

Experiments carried out by Brink and Wood ('58) show that *Mp* as a component of the variegated pericarp allele has no

effect either on pollen tube development or on growth and reproduction of the plant. Emerson ('17) had found that the variegated pericarp allele was transmitted normally through the pollen, and the results we obtained from a series of identical reciprocal matings between variegated heterozygotes and homozygous nonvariegateds within an inbred line confirm his observations.

It was also established in our experiments that yields of grain in heterozygous variegated and homozygous nonvariegated plants, which were otherwise closely comparable, are the same. The experiment on which the conclusion rests was well controlled, so there is little doubt concerning the validity of the conclusion drawn from it. A less precise, but probably adequate, test indicated that variegated pericarp in homozygous condition also is neutral in effect on yield of ears. It may be concluded, therefore, that growth and the reproductive capacity of the maize plant are the same whether or not *Mp* is present in the genome.

The dispensability of *Mp* to the maize genome raises a question that is critical for one aspect of the controller hypothesis, namely, uniqueness of function of controllers. According to the hypothesis, *Mp* as a component of a system of controllers should incite certain loci to perform their respective metabolic functions during plant development. Since *Mp* is an element that "acts at a distance" as well as locally (McClintock, '51; Fradkin and Brink, '56b), it might be presumed that the amplitude of the metabolic effects in this instance is relatively broad. The plant, however, grows and reproduces normally in the absence of *Mp*. The element, therefore, cannot be considered an essential constituent of the genome.

The fact of dispensability is compatible, on the other hand, with the assumption that *Mp* is involved in some general nuclear function performed by a series of relatively nonspecific chromosome elements that may substitute for each other. This view is reminiscent of Caspersson's hypothesis ('56) on the physiological role of heterochromatin.

THE DEVELOPMENTAL PATTERN RESULTING FROM *MODULATOR* ACTION

The essence of the developmental process is orderliness, the progressive unfolding in time and space of parts with determinate qualities, the different structures being smoothly coordinated at all stages as an organic whole. What principle, or set of principles, can account for the specific forms of reactions occurring invariably at each successive stage and maintenance of the individual as a moving equilibrium? Mutable loci, on first sight, would seem to be a quite hopeless place to look for clues to the basis of such functional harmony. For are these loci not the negation of regular behavior, elements of disorder intruding unpredictably into an otherwise orderly process?

Consider an ear of maize, as a familiar example. It is a slightly tapered, elongated, cylindrical structure on which are neatly inserted an even number of straight and parallel rows of repetitive units, the kernels. Now suppose the ear was borne upon a plant carrying the variegated pericarp allele. What then is the picture? The symmetry of the principal structure is strictly maintained, but superposed upon it is an assortment of red patches and streaks distributed in a disorderly fashion over the surface. One asks whether the process whereby this bizarre pigmentation arises is coordinate with the developmental mechanisms whereby the ear became radially symmetrical and the kernels were formed in regular succession. The burden of proof surely is on those who argue that there is a single principle at the basis of both ear form and pigmentation.

Yet, mutable loci display a measure of orderly action the character of which is illustrated by the dose effect of *Mp* in variegated pericarp plants (fig. 1). Medium variegated pericarp ears from individuals heterozygous for a colorless *P* allele in the 4Co63 inbred strain show about 81 mutations of variegated to red per 1000 kernels. This frequency may be considered the standard, or ground value, since these plants

Fig. 1 A, the medium variegated phenotype. This ear illustrates the typical expression of a variegated allele in single dose (heterozygous colorless pericarp). No transposed *Modulator* is present.

B, the light variegated phenotype. The variegated allele again is carried in single dose, as in the ear at the left, but one additional *Modulator* unit also is present, elsewhere in the genome. The latter *Mp* markedly reduces the number of mutations of the variegated allele to self-red, thus changing the collective phenotype from medium variegated to light variegated.

carry *Mp* only at the *P* locus, where the element is present in conjunction with the P^{rr} gene. Introduction into the genome of a single transposed *Mp* reduces the mutation frequency to 32 per 1000 kernels. The frequency is further sharply reduced to 4 per 1000 kernels in plants carrying two transposed *Modulators* (Brink, '54). Thus an effect of adding *Modulators* to the genome is to decrease at an accelerating rate the number of transpositions of *Mp* away from the *P* locus. Extrapolation of the observed mutation values indicates that a plant carrying a variegated allele plus four or five transposed *Modulators*

would be indistinguishable phenotypically from one homozygous for the standard P^{ww} allele (no *Mp* present) conditioning colorless pericarp and cob.

What is to be inferred from these data concerning the regulatory action of transposed *Mp* on the expression of pericarp color? The only deduction that is warranted in this case, in my judgment, is one of limited significance for the problem of differentiation. The nature of the basic event involved is the same in all three variegated genotypes, namely, transposition of *Mp* away from the *P* locus, thus permitting the gene for red to express its pigment-producing action. This transposition is sporadic throughout. Adding *Mp* elements to the genome only moderates the irregularity. An invariable effect of the mechanism is attained only if a sufficient number of *Modulators* are introduced to reduce the probability of transposition of *Mp* from the *P* locus to zero. Pericarp and cob would then be uniformly colorless.

Transposed *Mp,* in increasing doses, conditions the expression of variegated pericarp in a statistically regular way and, in this sense, generates patterns. But the basic event — transposition of *Mp* away from the *P* locus — remains random at all *Mp* doses in terms of distribution over the array of dividing cells in which it could occur. Thus *Mp* fails in an essential respect to yield patterns of the type characteristic of normal differentiation.

It might be argued that variegated pericarp is a "pathological" expression of controller action and that the evidence derived from it is therefore without probative value for the controller hypothesis. The relation of detectable point mutations, most of which are pathological, to progressive evolution is analogous. What is demanded in both instances, of course, is positive evidence validating the assumptions on which the respective explanations advanced rest. One of the basic conditions to be met by a theory of development is invariability of specific response by particular groups of cells to given sets of conditions, whether the latter are external or internal to the

chromosomes. The evidence thus far adduced in the case of *Mp* is not in accord with this requirement.

DISTRIBUTION OF *MODULATOR* TRANSPOSITION SITES

When *Mp* is transposed from the *P* locus in the mutation of variegated pericarp to red, *Mp* usually is "captured" at another chromosome site. Where does *Mp* go in its initial move from the pericarp locus? The answer to this question is instructive in terms of the potential role of such a factor in development.

Nancy van Schaik ('57), in our laboratory, tested 87 independently occurring transpositions of *Mp* in medium variegated pericarp maize. The new site of *Mp* was found to be linked with the *P* locus in 56, or nearly two-thirds, of the cases. The element assorted independently of *P* in the remaining 31 examples. One of the latter was linked with a reciprocal translocation marking chromosomes 4 and 5 and another was linked with T5–9a; the positions of the others were not determined.

Distribution of the new sites of *Mp* obviously is nonrandom. Using Rhoades' measurements ('50) of pachytene chromosome lengths in conjunction with appropriate crossover data, van Schaik estimated that if *Mp* were transposed at random it should be linked with the *P* locus on chromosome 1 in only about 10% of the cases. The tests show, however, that 64% of the transposed *Modulators* are so linked. Evidently there is a strong tendency for *Mp,* on leaving the *P* locus, to take up another position on the same chromosome less than 50 crossover units away.

The distribution of the 56 chromosome 1 sites in terms of distance from *P* also is informative. (The direction from *P* of the transposed *Mp* sites was not determined in these experiments.) In 38 of the 56 cases, less than 5% recombination between transposed *Mp* and *P* was observed. The frequencies fell off rapidly as the distance of transposed *Mp* from *P* increased. There were seven cases in which the recombination values lay between 5 and 10%, three between 10 and 15%, four

between 15 and 20%. Only four beyond the 20% level could be certainly established as linked.

The recombination value between transposed *Mp* and *P* was zero in 23 of the 56 cases of linkage. It is likely that the small family size on which some of the determinations were based is responsible for the failure to observe any recombination in these instances. No values as low as zero were observed in a subgroup of 24 more-extensively tested transpositions, although there were nine instances in this sample in which the recombination frequencies were less than 5%.

The evidence shows that, when *Mp* is transposed from the *P* locus, it tends strongly to go to one or another position nearby on the same chromosome. The frequency with which transposed *Mp* occupies any given position on chromosome 1 increases sharply as the distance from the *P* locus decreases. The result is that a high proportion of the transposed *Modulators* are clustered near the original site. The conclusion is inescapable that distance along the chromosome is a major factor determining the position to which *Mp* moves.

There are obvious difficulties in reconciling this conclusion with the hypothesis that transposition is a means whereby *Mp* regulates gene action during ontogeny. There is no basis for assuming, for example, that the various loci in the general vicinity of *P* are ordered in a way that is significant for action during development. Evidence showing that homozygous reciprocal translocations in maize are usually without major effect on the phenotype, in fact, argues directly to the contrary (Brink, '32; Roberts, '42).

When *Mp* moves away from the *P* locus it permits full expression at *P* of the P^{rr} gene for self-colored pigmentation. By analogy with its effect on pericarp color, *Mp* may be supposed to suppress, or otherwise alter, gene action at the new site to which it is transferred. If this is true, then each time the *P* locus is activated by transposition of *Mp* away from it another locus is inactivated by acquiring the element. Only if the old and new loci are physiologically complementary to each other

in some way would "turning on" one locus and "turning off" the other be meaningful for orderly development.

The present evidence implies that any one of several linked (and also unlinked) loci may be inactivated when the *P* locus is activated by transposition of *Mp*. The probability of such reciprocal activation and inactivation in the case of the linked loci is a function of the distance along the chromosome from *P*. There are no grounds for thinking that in the long chromosomal segment in question a corresponding multiplicity of loci occurs each of which is physiologically complementary to *P*.

MUTATION AND DIFFERENTIATION

Finally, there is a basic difficulty inherent in any hypothesis of development that assumes that mutation is a factor in differentiation. This long-familiar problem (see Sturtevant, '51) is seen in its full dimensions in the higher plants in which the distinction between germplasm and somatoplasm, in the Weismannian sense, is practically nonexistent. A mutation occurring in vegetative tissue need only arise in a cell lineage in which archesporial cells eventually are formed in order to be inherited. No difference between mutations at stable and unstable loci in this respect is evident. Thus mutations as genetic phenomena and as factors in differentiation come into direct conflict.

If it is assumed that ontogenetic differentiation rests in part on a process of intrachromosomal differentiation at the determinant level, then the evidence demands that a mechanism effecting intrachromosomal dedifferentiation also be postulated. The dedifferentiation must occur at the time of, or before, gametes are produced; otherwise, the fertilized egg, initiating the next ontogenetic cycle, receives a set of determinants that are not at the ground level but are already transformed. Such a mechanism of intrachromosomal dedifferentiation, invariably reversing the genetic changes occurring at the *P* locus during ontogeny, is not in evidence in the *Mp* data.

One aspect only of the controller hypothesis has been under consideration in this paper, namely, that relating to regulation of gene action during development. Except incidentally, reference has not been made to controllers as factors in mutation, a phase of the broad problem of organization of the genetic material in which McClintock's work on mutable alleles has been especially enlightening and fruitful. Some of the difficulties encountered in applying the controller concept to developmental physiology have been illustrated. Evidence for the significance of factors of the *Mp* class in mutation is both more extensive and more convincing and is to be appraised quite apart from the present discussion.

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OPEN DISCUSSION

BOYES [2]: I wonder about the relation between the *Mp* and suppressing genes or inhibitors. Of course I realize that there are very definite differences, since *Mp* does not seem to have a permanent position but moves in the nucleus. Also it seems to change its action with its position. Might there be a *Knob* or a heterochromatic region near your *P* locus; in which case, if the *Mp* moves in, the *P* heterochromatin influence would affect it?

BRINK: No cytological work has been done on *Mp* stocks in our laboratory. Dr. McClintock reported that *Ac* and *Ds* were

[2] J. W. Boyes, McGill University.

not morphologically distinguishable in her material. I might say that none of the recently presented data on mutable alleles is inconsistent with the idea that T. Caspersson has put forward in more or less a definite form. *Mp* may represent a class of chromosomal components (heterochromatin) that has some general function in development, such as the massive production, say, of simple proteins that later become differentiated under the action of specific genes. Now on the other point, it is true that *Mp* has different effects, depending on its location. The effect is a function of the locus at which the *Mp* is present. As far as we know, there is no distinctive action of *Mp* as such.

Ephrussi[3]: I tried to point out earlier all the difficulties that cytoplasmic geneticists have in distinguishing particles from other mechanisms that imitate particles. I think all the doubt we have there can be exactly transposed to the situation with *Mp*. You did not use the word "particle" but you were talking about entity — unitary element; I really think you meant a particle. You quoted certain arguments in favor of a particle hypothesis. One was mutability. We know now that mechanisms that do not involve macromolecular structure can imitate mutability. So none of your argument convinces me that you are dealing really with an insertion of a particle in the neighborhood of the locus.

Let us take Dr. Lederberg's work on *Salmonella* as an example. To interpret his first observations, he could have invented a particle that was being inserted next to the locus; but he did not. I think you have presented no evidence of a particulate element, and the only thing that would convince me that you are dealing with a particle inserted here and there would be the distortion of the chromosome (linkage) map. Until I have proof that you have such evidence I would still like to think of these changes as epigenetic changes. It means that the state of the locus itself is changed, and this change need not involve the insertion of a closely linked element.

[3] Boris Ephrussi, University of Paris.

It seems to me that you presented one piece of evidence that rather favors this interpretation: contrary to all real particles that we know about in the chromosomes, this one, as you said, is a dispensable one. So I think it rather favors local changes of activity. Against this you quoted an argument, namely, the unrelatedness of the functions of the different genes that are affected by the so-called insertion of the controlling elements. I wonder whether there are not a number of conditions that could affect the functional state of a number of genes, the functional relationships of which are not obvious to us. In the strictly Mendelian case of many suppressors there are comparable situations. In *Drosophila* there are many suppressors that function apparently by modifying in some very general way the intranuclear or intracellular condition, with the result that a number of genes with apparently unrelated functions are affected in their expression. I wonder whether it could not be a situation of general imbalance of this type.

Brink: Dr. Ephrussi's comment relates to the general point of what we mean by the symbol *Mp*. What does it imply? There is no morphological evidence showing that a chromosome containing *Mp*, or a like factor, is different from one that does not. I do not think there is any proof for the existence of an *Mp* as a particle of the sort that Dr. Ephrussi would accept. Precise linkage studies are not feasible with an element that frequently transposes. One cannot discriminate between crossovers and other movements. As I have demonstrated here, *Mp* often moves by very short steps. So we cannot get exact linkage data.

The one bit of evidence that suggests that *Mp* is a physical element rather than simply a shape that the chromosome segment takes when it contains *Mp* is this: The tests, of which I reviewed some 13 — five cases of transposed *Mp* in one inbred line and eight in another — showed the same class of effects thoughout on the variegated phenotype. These effects are not a function of the position of transposed *Mp* in the

genome but of *Mp* itself. Now I do not think this uniform action, independently of position, is understandable except on the assumption that *Mp* is a definite physical element.

AUERBACH [4]: I was rather convinced by the part by which Dr. Ephrussi was not convinced. I thought the evidence really showed that we are dealing with a particle, and I thought the part of the evidence that showed it was a particle is that it seems to have one, and only one, locus. When it disappears from one spot, it appears at one, and only one, other spot.

You think that you have evidence that *Mp* is a dispensable element. Was the chromosome with the absence of *Mp* really obtained by loss during a transposition? Or could you simply not detect *Mp* any more? In the latter case, it may still have been there in an inactive or somehow altered form.

BRINK: Dr. Auerbach has called attention to a shortcoming, but not a fatal one, in the experiment that we made. We did not have the stock at the time, but the test we should have made on the total developmental effect of *Mp* is a comparison between a red mutant from variegated and variegated itself. We did not have this pair of stocks at the time, so we used as control a red cob, colorless pericarp strain isogenic with the variegated stock except with reference to loci closely linked to *P*. The results are still meaningful.

AUERBACH: I wonder whether a red mutant would satisfy this condition. It would need to be a mutant that occurred in a twin spot.

BRINK: The desirable type of red mutant would have been one from a twin spot that, in a test cross with *Ds*, would not promote the *Ds* type of chromosome breakage. That is the criterion for the presence of *Mp* in a genome at any locus. If the *Ds* type of chromosome breakage is promoted we conclude that *Mp* is present. We would have chosen, then, a red mutant that was negative in this respect for comparison with the parent medium variegated.

[4] Charlotte Auerbach, Oak Ridge National Laboratory; on leave from University of Edinburgh.

STERN [5]: I think Dr. Brink has done a great service in separating the *Mp* phenomenon into two parts; namely, the mutable gene part and the ontogenetic one. Drs. Brink and McClintock have done a marvelous job in demonstrating that mutable genes in corn are under the influence of something that seems to be similar to heterochromatin of other organisms and that can control the action of genes.

With regard to the ontogenetic problem, I believe that we should not look for control of genic action by special genic agents as the primary event. Ontogeny, as Dr. Ephrussi pointed out in his presentation, is an epigenetic phenomenon. If we remind ourselves of embryonic induction and regional differentiation, then it is clear that it is the position of parts in the egg or embryo that initiates their local ontogenetic pathways. Thus the control of this initiation is not within the nucleus. We might be inclined to say that these superimposed conditions are the agents that activate certain genes here and deactivate certain genes elsewhere. This way of speaking is, however, misleading.

"Activation of a gene" implies an event at the gene locus in a sense that truly applies to the *Mp* situation. In ontogeny, however, apparent activation of a gene may well be an event that occurs far removed from the gene itself. It might be best at present to avoid the term gene activation and speak only of epigenetic realization of gene action. Unless we actually know that an observed ontogenetic event is caused by release of activity of an earlier less-active or inactive gene, the analogy between *Mp* effect on gene action and epigenetic realization of gene action may be a hindrance rather than a help in our understanding.

EPHRUSSI: Earlier, when I talked about epigenetic effects, I precisely had in mind nuclear epigenetic effects that do happen at or very near the gene level. I did not talk about very remote effects. If for me a typical example of an epigenetic effect were the Himalayan rabbit, I would not have used a

[5] Curt Stern, University of California, Berkeley.

new word because we have an old one — phenotype and phenotypic variation (which is immediately reversible).

BRINK: As I understand the controller hypothesis, it has been put forward in the terms to which Dr. Ephrussi has just referred, as activation involving an event at or near the locus whose effect is altered.

BRAUN [6]: I concur, of course, with Dr. Stern's suggestion that the type of effect that Dr. Ephrussi describes as epigenetic may be of major importance in ontogeny. But I also wonder if at times we do not tend to interpret complex phenomena on merely one basis. When it is superimposed on one type of mechanism, we may be dealing with additional mechanisms. One possible contributing mechanism may involve selection of specific cell types from among many that can occur spontaneously in ontogeny. In this respect I wonder whether, despite Dr. Brink's skepticism of the role of his *Mp* in ontogeny, there might not be a potential role for such a mechanism.

S. Zamenhof, in some rather interesting studies on bacterial cell populations, demonstrated that, when gene instability occurs, a mechanism assuring selective establishment of specific cell types under conditions where they ordinarily would not occur is also provided. This is so because instability provides an enormous amount of raw material for selection. Without in any way attempting to generalize from this as to a major ontogenetic mechanism, I would like to raise the question whether, under circumstances where you are dealing with the selection of specific cell types, a mechanism like your *Mp* may not play a role in developmental differentiation.

BRINK: I should like to be understood as having said only that no critical evidence in favor of the controller hypothesis as a mechanism of ontogenetic differentiation can be derived from the variegated pericarp data. Much of Dr. McClintock's pertinent work is unpublished; when available, it will provide a broader body of evidence on the question at issue.

[6] Werner Braun, Rutgers University.

I am not prepared to deny, Dr. Braun, that we may be at the beginning of this story, not at the end. There may be a great deal more involved in these phenomena from the standpoint of chromosome organization than has as yet been discovered. I should like also to say with reference to the earlier remarks of Dr. Stern that we can make one or the other of two assumptions with reference to the action of these elements at a locus. We can suppose that the chromosome is wound up like a clock and is set to go off in particular ways by virtue of its inherent organization, or that changes in state or transposition occur in response to some environmental circumstance. In the latter case, of course, we simply move the developmental problem back one step. I have been inclined to interpret certain of Dr. McClintock's published statements as implying that she favors the clock-type process. Perhaps I am mistaken in drawing that conclusion.

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CORRELATION OF CHROMOSOMAL AND PHYSIOLOGIC CHANGES IN TUMORS

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SEVEN FIGURES

The individual organism accepts two risks for the evolutionary advantages of genetic flexibility: disease inherited through the germ track, and the pathological consequences of somatic cell variation — foremost among them, cancer.

The Mendelian approach to neoplasia centers on predisposition and resistance in cancer origin. The somatic approach, hindered by the elusiveness of initiation at the cellular level, concerns itself with nuclear diversity as the basis for continuing change in established tumors. Treating malignant tissues like microbial populations, it relies on statistical analysis of variance in a given physiologic response after experimental exposure to selective pressures designed to alter this response. It attempts to distinguish between physiologic adaptation and genetic alteration by the randomness, heritability, and stability of new traits.

Current methods in tissue genetics cannot differentiate between true gene mutations and other causes of phenotypic change in the often aneuploid nuclei of cancer cells. There is every reason to assume that neoplastic genotypes fluctuate within very wide limits set by point mutation (Klein and Klein, '55; Russell and Major, '57; Klein *et al.*, '57), mitotic crossing over (Carter, '52), recombination, and gross chromosomal changes (Hauschka, '57). The last are most accessible to analysis, but inspection of chromosomes affords only a furtive glimpse of the genetic skeleton in the somatic closet. Assignment of a specific function to a definite chromosome

constellation in so variable, yet so integrated, a system as the cancer cell rests entirely on circumstantial arguments. Certain functional consequences of heteroploidy are, however, sufficiently consistent to suggest correlation with chromosomal imbalance.

Emphasis in present-day cancer cytology has shifted from the etiologic preoccupation of early workers (Winge, '27, '30) to a physiologic morphology of neoplasms. What is the nature, frequency, and stability of nuclear changes in malignant cells? How permanent are the stem lines responsible for the growth advantages of cancers, and how viable the products of their mitotic errors? Which clear-cut functions of somatic cells may be investigated in relation to the karyotype? Among such properties are resistance, immunologic specificity, and growth characteristics.

Ascites tumors have proved to be particularly favorable for measurements of cytogenetic variation in cancer. Many ascites tumors of mouse, rat, and man have chromosome numbers and types differing from the diploid idiogram of the host species (see Koller, '47, '56; Hauschka and Levan, '51; Bayreuther, '52; Makino and Kanô, '53; Tjio and Levan, '56; Hansen-Melander *et al.,* '56). This applies also to primary solid tumors, where nuclear analysis presents far greater technical difficulties. Spectrophotometric measurement of nuclear deoxyribonucleic acid (DNA) content (Leuchtenberger, '57; Mellors, '55) is a good survey tool but no substitute for direct observation of chromosomes, which is more perceptive of karyotypic subtleties in their genetic bearing on functional individuality.

Minor anaphase disturbances, multipolarity, endomitosis, and endoreduplication are perpetual sources of flux in the neoplastic cell population. So-called "cryptostructural" changes attributable to translocations and detectable only through length measurements (Levan, '56a,b) contribute further genotypic rearrangements as the raw material for selection. Genetic change produced by this karyotypic drift is a *sine qua*

non in tumor progression (Foulds, '54) and autonomy, regardless of its disputed role in the genesis of tumors.

In the absence of meiosis as a stabilizer, the host environment is the only restrictive influence on the mosaic of genotypes that diverge from the tumor stem line. Instability of transplanted tumors can, however, be minimized by uniform transfer conditions. These should be immunologically neutral and should avoid differences in strain, sex, and age of host, and in dilution media and cell dose. Storage of frozen glycerolized cell suspensions at —76°C. between experiments is an additional safeguard against unpredictable fluctuations. Regular periodic deposits of valuable tumor lines in our

TABLE 1

Frequency of nuclear shapes and number of lobes in three lines of mast-cell tumor P815 [a]

LINE	COMPACT NUCLEI	LOBATED NUCLEI	RING NUCLEI	RIBBON NUCLEI
	%	%	%	%
P815–DON–S	80.8	19.2	0	0
P815–DON–RI	21.4	70.6	5.2	2.8
P815–DON–RII	79.0	21.0	0	0

[a] 500 cells were classified for each subline.

frozen-tissue bank protect the experimental investment that goes into development and comparative study of such lines. The marker properties of Potter's mast-cell tumor P815–DON–RI (see table 1) are a good example. This mouse tumor is a pseudodiploid with a sharp mode at 40 but with two new chromosomes present in every cell; its nuclei are lobated; the surface is characterized by antigen D; it is less virulent, and produces considerably less serotonin and histamine than related sublines. It also secretes heparin and hyaluronic acid, and its transplantation specificity is in keeping with the isoantigens of the DBA/2 mouse strain in which it originated. Finally, it is resistant to the purine antagonist DON (6-diazo-5-oxo-L-norleucine).

This attempt to relate such functions to the karyotype is not meant to be a full review. It includes discussions of topics we have investigated, often in collaboration with Drs. Klein, Levan, Hoecker, and Amos. It is divided into sections dealing with the influence of heteroploidy on growth, resistance phenomena, and antigenic differentiation. New data on the cytology of drug-resistant sublines and isoantigens of tumor clones are here presented for the first time.

GROWTH CHARACTERISTICS AS INFLUENCED BY POLYPLOIDY

The contradictory beliefs about growth advantages or disadvantages inherent in polyploidy are based on superficial examinations of tumors that are not really comparable. A critical answer to this question demands measurements on related sublines derived from the same neoplasm and differing only in their chromosome ploidy. Collaboration with Révész and Klein (Hauschka *et al.,* '57) has given data on what happens to growth rate when the nuclear constitution and individual cell volume are doubled.

Which is most influential as a check on cell multiplication: total cell number attained, total mass, or total surface exposed to the host? Polyploidization generally does not alter nucleocytoplasmic ratios, but the compensatory homeostasis of cell mass involves a surface increase by a factor of only 1.6 rather than 2. Furthermore, doubling of the chromosome number could alter gene dose effects, some of which are less cumulative than others. This may change the synthetic potential per unit volume of protoplasm.

Our growth analysis of the hyperdiploid Ehrlich ascites tumor ELD and its polyploid derivative ELT has profited from enzyme data for the same material. Cytochromes b and c are identical per unit volume, i.e., the polyploid cell contains twice the amount of respiratory enzymes as the corresponding diploid (Britton Chance, personal communication). Similarly, the aminopeptidase values determined by Patterson and Podber ('56) have a 2:1 ratio and are exactly

proportional to the ploidy of the two cell types. Identical availability of these and other important enzymes seems to bestow equal synthetic potential, as shown most convincingly by the increase of cell volume in the absence of mitosis. Cell growth of ELD and ELT was compared by Révész (Hauschka *et al.,* '57) during a 3-day amitotic period after irradiation. Calculations from the slope of the regression lines showed that the mean volume of the hyperdiploid cells increases 1.37% per hour and that of the hypertetraploids 1.38% per hour. In absolute values, this means the synthesis of 15.3 μ^3 of new protoplasm per hour per ELD cell, and 31.6 μ^3 per hour per ELT cell.

Under normal conditions of growth, i.e., when mitosis is not experimentally inhibited, the ELD and ELT should therefore be expected to multiply at exactly the same rate. During the initial rapid growth phase after inoculation of one million cells per mouse, ELD and ELT cells doubled at the same rate until environmental limitations began to retard further synthesis of neoplastic substance. This happened earlier for ELT than for ELD. The total cell number attained prior to the death of a 25-g Swiss mouse averaged about two billion hyperdiploid cells as against only one billion hypertetraploid cells. Terminal values were closely reproducible in several repeat experiments. On the other hand, the lethal tumor mass that accumulated in a mouse of given strain and weight was the same for both tumors. Thus total neoplastic mass rather than cell number, as such, or surface is the fundamental determinant of growth limits; and there is no intrinsic difference in growth capacity between comparable diploid and polyploid cancer cells.

CHROMOSOMAL IMBALANCE AND VIRUS SUSCEPTIBILITY

Neoplastic tissue cultures and ascites tumors have proved excellent media for analysis of relations between cells and viruses. Much of this work has been motivated by the clinical aim of selective viral oncolysis (Moore, '52). Other investi-

gations were concerned with cytopathogenic changes in the infected host cells and quantitative differences in virus yield between normal and neoplastic tissues (Koprowski, '56; Cassel, '57a, b; Syverton, '57).

Prominent among tumor-necrotizing viruses are Mengo, Bunyamwera, West Nile, WS (neurotropic) influenza, and IHD vaccinia. Most of these oncolytic agents are also neurotropic, but certain neurotropic viruses (e.g., eastern and western equine encephalitis, Semliki Forest, herpes, fowl pox, and poliomyelitis) gave consistent negative results regardless of the tumor type used as medium.

The cytopathology of infected cancer cells (Orsi *et al.*, '57) reveals little about specific intracellular localization of these viruses. The small size of Mengo and other viral particles has defeated their electronmicroscopic identification in the neoplastic host cells. Inclusion bodies visible with the light microscope are rare. Complete nuclear destruction is preceded by margination of the chromatin, appearance of parachromatin bodies, and multiple nuclear budding. Karyorrhexis is accompanied by a striking increase in the cytoplasmic lipids, and by impaired activity of the respiratory enzymes.

A curious, and probably spurious, relation between chromosome constitution and virus susceptibility was observed by Koprowski ('56). He tested ten viral agents against each of ten histologically diverse ascites tumors. The modal chromosome numbers and the range of nuclear variability in this material had previously been determined (Bayreuther, '52; Hauschka and Levan, '53; Tjio and Levan, '56): I checked these again in the ascites lines available for this work. The Mengo, Bunyamwera, and West Nile viruses proved oncolytic for the six most heteroploid neoplasms; they failed, however, to damage three near-diploid mouse tumors. No virus has yet been found capable of multiplying in the near-diploid Yoshida rat sarcoma.

Perhaps more significant than those differences between unrelated growths are Koprowski's results ('56) with the diploid lymphosarcoma 6C3HED (virus-refractory) and its

three Swiss adapted heteroploid sublines (very susceptible to viral oncolysis). At least one of these sublines, however, analyzed by Feldman and Sachs ('58) and erroneously labeled as "6C3HED," has in our opinion both the chromosome number distribution and the characteristic marker chromosomes (two minutes, one metacentric, one telocentric with secondary constriction) of ELD. The latter is highly susceptible to virus. Laboratory contamination and replacement of the 6C3HED ascites by ELD cells must, therefore, be ruled out before altered virus susceptibility in the "Swiss adapted 6C3HED" can be claimed.

It should be emphasized that certain viruses can be propagated in tissue cultures of diploid cells. Polio viruses grow well in primary cultures of monkey kidney, where K. H. Rothfels and L. Siminovitch (personal communication) find the normal diploid Rhesus number of 42 chromosomes in 98% of the cells. On the other hand, most of the permanent cell strains serving as successful virus media have superficially triploid or near-tetraploid nuclei (Syverton, '57). X-ray-induced giant cells that continue to metabolize abundantly, despite their damaged genetic apparatus, exhibit "an enormously increased susceptibility to virus invasion" (Puck, '57).

Impaired equilibrium of the karyotype may alter cytoplasmic constituents and surface properties, thereby rendering the host cells more susceptible to viruses. One might speculate further, that the abnormal enzymic integration of aneuploid cells enables certain viruses to prosper on metabolic surplus for their own biosyntheses. It would, therefore, be instructive to learn more about the nuclear constitutions of resistant mutants surviving in a partially oncolyzed ascites population, and about the chromosomes in Puck's virus-refractory, yet virogenic, tissue cultures.

HETEROPLOIDY AND RADIATION SENSITIVITY OF TUMOR TISSUE

The differential radiosensitivity of normal diploid tissue types is well established, germinal and hemopoietic cells

being most susceptible, muscle and nerve most refractory. Basal oxygen consumption may be among the more important variables influencing radiation response.

There are as yet no morphologic or functional criteria permitting a reliable forecast of radiation sensitivity from pathologic biopsy material. It has been taken for granted that polyploid cancer cells are more resistant to X rays than diploids, hence responsible for tumor recurrence after initially successful X-ray therapy (Koller, '47). Where polyploidy is balanced, and the chance injury to vital genes accordingly reduced, decreased radiosensitivity has been experimentally substantiated. For example, Sparrow and his collaborators demonstrated higher radiation tolerance in polyploid than in diploid plants (Sparrow, '56). Polyploid and diploid yeasts are less sensitive than haploids to ultraviolet and to ionizing radiation of all kinds (Tobias, '52). Haploid cultures would, of course, exihibit obligate susceptibility to recessive lethal damage, whereas balanced polyploids are protected by their multiple genetic spare parts. This obvious logic does not hold entirely true even among yeasts; Mortimer ('57) has found increased sensitivity in some higher polyploids. Quoting observations in a number of biological systems that show that chromosomal abnormalities increase in proportion to ploidy, he concluded that X-ray-induced dominant lethality in the higher-ploidy classes of yeasts "was associated mostly with chromosomal disturbances."

Precise chromosome studies of recent years make it certain that "tetraploid" tumor nuclei rarely contain exact duplicates of the diploid species idiogram. They may be monosomic or polysomic for entire individual chromosomes (Yerganian, '56) and perhaps similarly unbalanced with regard to deletions or translocations (Levan, '56a, b). The deficient portions of their genome would be as vulnerable to ionizing radiations as are haploids. Hence there is no uniform cytogenetic basis for a consistent correlation between polyploidy and radiation resistance in tumors, as borne out by contradictory published data.

A hypertetraploid cell population was isolated by Révész (Hauschka *et al.*, '57) after he inoculated 20 million irradiated ELD cells (4000 r *in vitro* in an N_2 atmosphere). No doubt, many of the irradiated cells were nonviable; thus the low cell dose prerequisite of Kaziwara's immunoselection experiments ('54) were reproduced and were probably responsible for differential survival of polyploids. After mitotic activity in the same hyperdiploid material and a polyploid subline was stopped by irradiation (1250 r), growth of cell volume in both proceeded at about the same rate. There was apparently no differential injury to protoplasmic synthesis in the two ploidy classes, and no correlation between cell volume of diploid and tetraploid cells and radiosensitivity.

In a recent attempt to alter the usual proportions of naturally occurring tetraploid cells in the near-diploid DBA/2 mouse lymphoma ascites by irradiating the donor animal with 2500 r, we obtained negative results (T. S. Hauschka, M. Reinhard, and J. T. Mitchell, unpublished). All the normal recipient mice inoculated with one million irradiated cells became leukemic within 2 weeks. There was no change in the modal chromosome number of 44, or in the frequency of the naturally occurring tetraploid population component, which was less than 3% both before and after irradiation. We obtained similar results with two other mouse lymphomas. Clearly, there had been no X-ray selection favoring the polyploids.

Koller ('56) found an increased frequency of diploid cells after treatment of human ascites carcinomatosis with radioactive colloidal gold. He suggests two possible interpretations of this unexpected result: either the diploid stem cells were more resistant to Au^{198} than aneuploids, or they were exfoliated into the ascites from the predominantly diploid local metastatic adhesions.

Puck and Marcus (Puck, '57) obtained an LD_{50} of 96 r in tissue cultures of polyploid HeLa cells and also for recent explants from three diploid normal human organs. The ex-

tensive genic asymmetries of HeLa cells may provide the reason for their surprising sensitivity.

Experimental selection of X-ray-resistant cells was reported by Schubert ('54). He injected suspensions of the ELD mouse tumor intramuscularly into both hind legs, where they formed solid tumors. The tumor on one side was irradiated; the other served as control. Both tumors were further transplanted, and unilateral irradiation was continued until 28,000 r was accumulated. Schubert's treatment resulted in significantly reduced radiosensitivity, and in a lessening of mitotic disturbances, but Lettré ('56) observed no decline in susceptibility to nitrogen mustard and other drugs. Unfortunately, the chromosome constitution of the derived line was not determined.

TUMOR KARYOTYPES IN RELATION TO ALTERED DRUG RESPONSE

Analogies between microbial heredity and the somatic genetics of malignant cell populations are especially apparent in studies of drug resistance. It is a severe handicap for the progress of such work that experimental recombination by cell fusion, transforming substances, or transducing agents has not yet been accomplished in mammalian tissue cells (Lederberg, '56).

Changes in the drug susceptibility of cancer cells have on the whole been random, stable, irreversible, and heritable (Law, '56). Luria and Delbrück's fluctuation test ('43) was applied by Law ('52) to interpret the origin of A-methopterin resistance in a lymphocytic neoplasm. This resistance arose independently of exposure, responded to treatment in stepwise fashion (similar to penicillin resistance in microorganisms), and persisted for long periods in the absence of the antagonist. Law's data therefore implicate mutation and selection, rather than physiologic adaptation, as the resistance mechanism. No clear case of physiologic adaptation that would preclude a genetic interpretation has yet been

encountered among numerous mouse tumors in Law's wide experience and that of others with growth inhibitors.

At present, the hypothetical mutants cannot be defined in precise genetic terms, despite considerable insight into their biochemistry (Nichol, '57). Mutant sublines often differ from the sensitive parent tumor not only in their reaction to the selective drug, but also in collateral, sometimes unrelated, functions. They may also exhibit gross morphologic innovations, such as increased nuclear lobation or cell size (fig. 1A, B, C). It therefore becomes important to determine whether certain refractory sublines are not simply clones with new chromosome combinations. Such variants are always abundant in the broad cytogenetic profiles of some cancers (Hauschka and Levan, '53; Levan, '56a, b). Grossly rearranged somatic genotypes, which constantly arise through mitotic anomalies, may respond to one-step selection. Indeed, rapid one-step selection by drugs, similar to streptomycin resistance in bacteria, also occurs among tumors (Potter and Law, '57).

Drug resistance has been described for the near-diploid Yoshida sarcoma (Hirono and Yokoyama, '55) and for very aneuploid rat hepatomas (Yoshida, '57). It occurs in the hyperdiploid Ehrlich ascites carcinoma (Lettré, '56) and in near-tetraploid Ehrlich lines (Gross *et al.*, '57; Jacquez, '57), as well as in mouse lymphomas (Law, '56), many of which have approximately diploid nuclei (Hauschka and Furth, '57).

Nevertheless, it is a widespread opinion that polyploid tumors are intrinsically more resistant to chemotherapeutic agents than diploids. This belief is not supported by controlled experimental comparisons; it rests on isolated findings, e.g., Yoshida's work with rat ascites hepatomas ('57). Among four liver tumors, each induced in an individual rat, only one (AH 130) was inhibited by nitrogen mustard treatment. The sensitive cell population happened to have a near-diploid modal chromosome number; the three resistant tumors were decidedly more heteroploid, with modes of 49, 67, and 70 chromosomes. These lines resisted triethylene thiophosphoramide, nitromin, and nitrogen mustard, but all, including AH

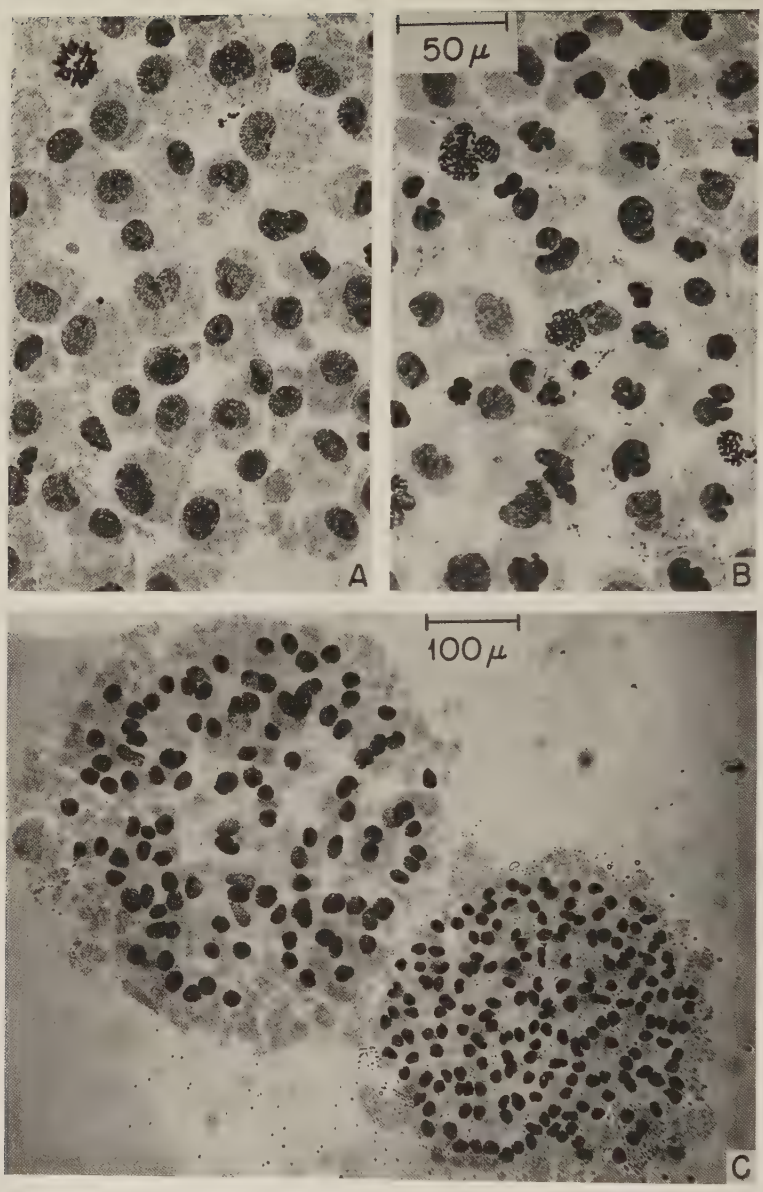

Fig. 1 A. Acetic orcein squash of mast-cell ascites tumor P815-DON-S; 81% of the nuclei are compact, spherical, or kidney shaped. The DON-R subline P815-DON-RII has essentially similar nuclei.

B. Acetic orcein squash of mast-cell ascites subline P815-DON-RI, in which over 70% of the cells have lobated nuclei.

C. Clonal islands in human ascitic fluid tapped from a patient with an ovarian carcinoma. Nuclear sizes show two classes as also suggested by chromosome counts in the same material. Acetic orcein fixation.

130, were sensitive to 6-mercaptopurine. In other words, the apparently greater resistance of the heteroploid cells to certain radiomimetic mutagens does not apply to antimetabolites that interfere with nucleic acid synthesis.

The difference in the response of heteroploids to two classes of compounds is interesting. Resistance of polyploids to mutagens capable of chromosome breakage may simply involve added protection given by supernumerary chromosomes against the random damage to essential gene regions. Resistance to a purine antagonist, on the other hand, would require specific mutations relevant to the affected metabolic pathways.

Chromosome conditions in Yoshida's several rat hepatomas allow no critical correlation between ploidy and drug susceptibility, since the tumors originated in different rats. A subline comparison was carried out by Hirono and Yokoyama ('55), who investigated two branches of the near-diploid Yoshida sarcoma, sensitive and resistant to the N oxide of nitrogen mustard. They found no change in chromosome number or selection of new chromosome types associated with resistance. This is, to our knowledge, the only published cytologic analysis of nuclear constitution with reference to altered chemotherapeutic response. Since broader coverage of this subject is desirable, I undertook chromosome comparisons of the following groups of related, sensitive, resistant, and dependent mouse ascites tumors: (1) an Ehrlich carcinoma, resistant to N-methylformamide, derived by Gross *et al.* ('57) from our hypotetraploid sensitive line and returned to me for chromosome analysis together with the sensitive stock tumor; (2) plasma-cell neoplasm 70429 (Potter and Law, '57), sensitive to A-methopterin, a resistant line 70429-AM-R, and a dependent line 70429-AM-D growing continuously in the presence of the drug (Potter, unpublished); (3) lymph-node leukemia P288; AM-S, A-methopterin-sensitive line and AM-RII, A-methopterin-resistant line (Potter, '58); (4) mast-cell tumor P815, line A sensitive to DON and two different resistant lines P815-DON-RI and DON-RII (the

cytopathology of this mast-cell neoplasm was described by Dunn and Potter, '57); (5) lymphosarcoma P1798, sensitive to cortisone and its resistant derivative P1798-CORT-R, developed by Lampkin and Potter ('58).

I am greatly indebted to Dr. Michael Potter of the National Cancer Institute for the several lines of tumors numbers 2 to 4, which he had converted into the ascites form, and for much pertinent information regarding their physiologic characteristics. Dr. Julia Lampkin kindly supplied the two branches of lymphosarcoma P1798.

The carcinostatic compounds used in selection of the lines mentioned function largely through interference with nucleic acid metabolism. A-methopterin inhibits C^{14}-formate incorporation into nucleic acid purine and thymine in resistant leukemic cells, but accelerates *de novo* synthesis in cells dependent on the antagonist (Skipper *et al.*, '52). Similarly, N-methylformamide (NMF) and DON interrupt purine formation (Barclay and Garfinkel, '57; Jacquez, '57; Karnofsky and Bevelander, '58). Cortisone blocks growth by derangement of nitrogen and carbohydrate metabolism and breakdown of tissue protein, especially in lymphoid tissue (Lampkin and Potter, '58). Through its interference with host immune

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Fig. 2 A. Metaphase from the plasma-cell neoplasm 70429-AM-D, showing 40 telocentric chromosomes, indistinguishable in size, structure, and number from the normal diploid chromosome set of the mouse. This cell furnished the idiogram for figure 6B.

B. The metaphase chromosomes in the stem cell of plasma-cell tumor 70429-AM-S.

C. Near-tetraploid metaphase typical of lymphosarcoma P1790-CORT-S; about 78 chromosomes, including five or six minutes, may be counted.

D. Metaphase of the lymphosarcoma P1790-CORT-R, showing the modal number of 41 chromosomes. The relatively small cytoplasm of these cells does not permit chromosome spreads without some overlapping.

E. Metaphase plate of the mast-cell tumor P815-DON-S (line A), showing the modal number of 41 chromosomes. The large equal-armed metacentric characteristic of this line is indicated by arrow.

F. The 40 stem-line chromosomes of the mast-cell tumor P815-DON-RI include two markers, an extra-long and an unequal-armed large metacentric, indicated by arrows. The markers can be easily identified in most well-spread metaphases. This cell supplied idiogram D in figure 6.

responses, it may indirectly promote survival of the more antigenic components of neoplastic cell populations growing in cortisone-treated animals.

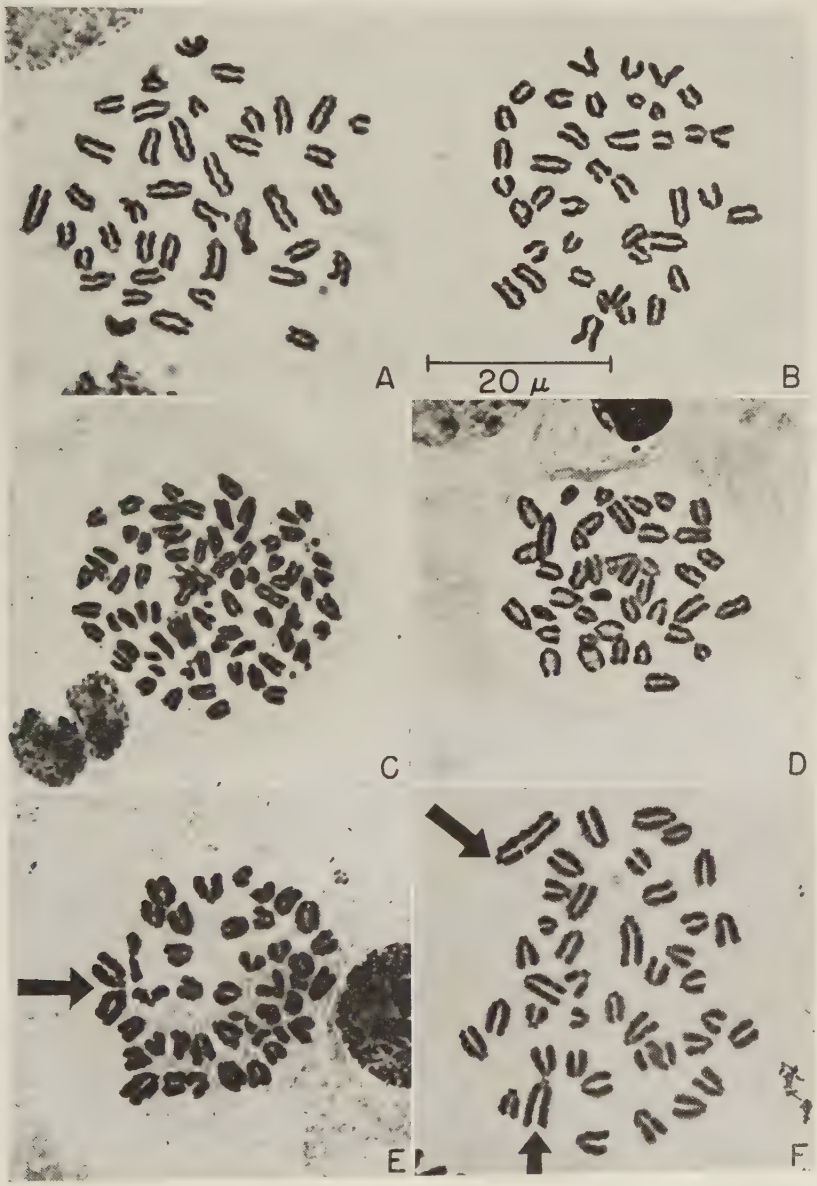

Figure 2

Even before precise chromosome counting is begun, attention to nuclear shape and size may reveal surprising differences. For example, the frequency of compact and lobated nuclei differed significantly in sublines of mast-cell tumor P815 (fig. 1A, B; table 1). This divergence pertained to the lines DON-S and DON-RI; since it was not shared by line DON-RII, it is either unrelated to resistance or indicative of two types of resistance. Likewise the obvious nuclear size discrepancies between lymphosarcoma P1798-CORT-S and -R should not be ascribed to direct selection by cortisone. The sensitive line had 99% near-tetraploid cells, whereas P1798-CORT-R contained 88.6% near-diploids, with a modal chromosome number of 41 (fig. 2C, D). This is an excellent example of a regulatory host mechanism superimposed upon those variations in the cell population that are amenable to drug selection. The sensitive lymphosarcoma seems to have been diploid originally, and now grows much more slowly than the cortisone-resistant line. The low growth rate enables the host to form antibody of effective titer against the tumor. A strong histiocytic and macrophage immune reaction was indeed apparent during the second week of growth. In Feulgen-stained preparations, macrophages were laden with ingested nuclear material; this defensive response favors immunoselection of polyploid elements (Kaziwara, '54; Hauschka *et al.*, '56, '57). By contrast, the ploidy distribution of the more virulent cortisone-resistant line P1798-CORT-R has remained unchanged. Its growth is too rapid for achievement of effective antibody titers that were further hampered by intermittent cortisone treatment. This can be documented histologically by the absence of an inflammatory response in Feulgen-stained smears. A difference in virulence may thus indirectly engender differences in chromosome constitution that are completely unrelated to the origin of resistance. Lack of correlation between altered antimetabolite response and virulence, as judged by mouse survival time, is evident from table 2.

TABLE 2

Lack of correlation between altered antimetabolite response and virulence [a]

MOUSE TUMOR LINES	VIRULENCE	REFERENCE
Lymphocytic neoplasm L1210-S and 6-thioguanine-R	S = R	Law, '56
L1210-S and 8-azaguanine-D	S > D	Law, '56
Mast-cell tumor P815-S and DON-RI and -RII	S = RII; S, RII > RI	Potter, unpublished
Ehrlich ascites-S and -NMF-R	S = R	Gross *et al.*, '57
Lymph-node leukemia P288-S and -AM-RII	S = R	Hauschka, unpublished
Lymphosarcoma P1798-S and -CORT-R	S < R	Lampkin and Potter, '58
Plasma-cell leukemia 70429-S and -AM-D	S < D	Potter, unpublished

[a] Virulence is judged by mouse survival time.

In order to obtain large numbers of exactly countable metaphase plates, we injected 10–15 μg of colchicine (2 μg/ml of mammalian Ringer's solution) less than one average cell generation time before fixation. This timing avoids numerical artifacts caused by spindle anomalies. Our previous work with ascites tumors pretreated and untreated with colchicine established the reliability of counts after judicious use of colchicine (Hauschka and Levan, '51, '58; Levan and Hauschka, '53b; Levan, '54).

Some tumors in the present series were more favorable for cytologic study than others. The relatively small cytoplasm of the tetraploid lymph-node leukemia P288 impeded metaphase spreading in acetic orcein squash preparations. Although exact counts were difficult, a modal number of about 80, including one or two minute chromosomes, was found in the stem lines of P288-AM-S and -AM-RII. Lymphosarcoma P1798 presented similar obstacles, as can be seen from the overlapping chromosomes in figure 2C, D; but the pronounced difference in ploidy distribution was easily determined from 1000 metaphases without recourse to exact counts. Numerous well-spread metaphases were the rule in preparations of the malignant plasma-cell tumors (fig. 2A, B and 3C, D), the mast-cell tumor (fig. 2E, F and 3A, B), and the Ehrlich ascites (fig. 3E, F and 4A, B). Chromosome numbers for the various

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Fig. 3 A. Endoreduplication in the mast-cell tumor P815-DON-RII. Stem cells of this line resemble those of the sensitive parent tumor; the equal-armed large metacentric (arrow) marker, like the other chromosomes, is four stranded.

B. The 40 metaphase chromosomes of this plate from the mast-cell tumor P815-DON-RI include the extra-long and unequal-armed metacentric markers (arrows).

C. Endoreduplication in the plasma-cell neoplasm 70429-AM-S occurring with equal frequency in the AM-S, AM-D, and AM-R sublines.

D. Apparent somatic pairing in an originally diploid malignant plasma-cell tumor of 70429-AM-D, consequent to successive chromosome doubling (endoreduplication) and preceding tetraploidy.

E, F. Stem cells of the NMF-R Ehrlich ascites in metaphase (E) with 72 chromosomes, and in anaphase (F). Arrow points to the extra-long marker chromsome.

sublines of the latter three tumors are compared in the histograms of figure 5A, B, C, based on exact counts in 750 metaphases.

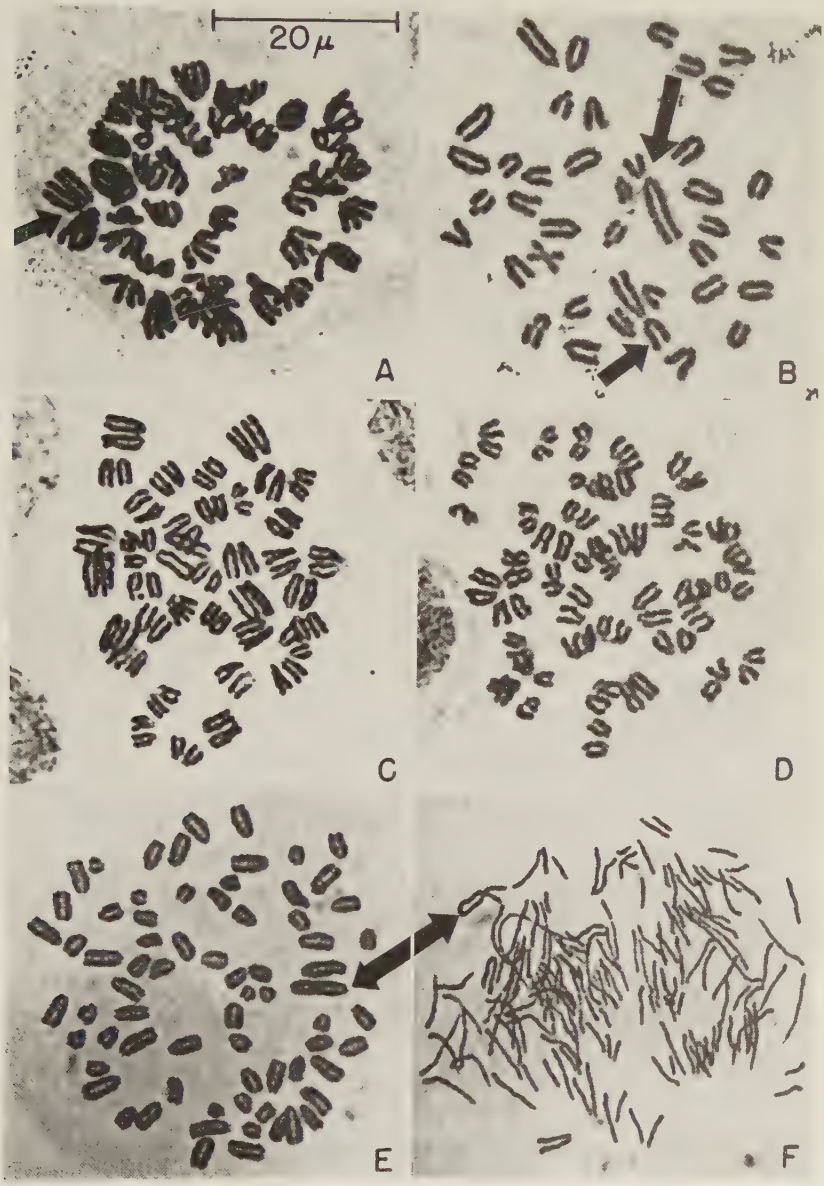

Figure 3

The NMF-R Ehrlich ascites showed a significant numerical departure from the sensitive parent tumor. Its low mode of 72 matched that of our most hypotetraploid clone isolated from the same tumor (Hauschka, '53a, b; Hauschka and Levan, '58). Of the resistant cells, 81% contained a prominent long marker chromosome (shown by arrows in fig. 3E, F and 4B). This long unit was absent from all but 3% of the sensitive cells in which minutes were unusually frequent (fig. 4A).

Were it not for the identity of the chromosome number distribution in mast-cell tumors P815-S and P815-DON-RII (fig. 5B), the steeper mode of the other resistant line, P815-DON-RI, and its two very distinct marker chromosomes might be considered characteristic of resistance as such (fig. 2F and 3B).

The only permissible conclusion is, however, that these new chromosomes contain genes of considerable survival value for the RI mutant; hence they are present in almost every cell, though not necessarily linked with DON resistance.

There are several functional similarities in the P815-S and RII lines, but altered in RI, and matching the difference in nuclear morphology and chromosome number distribution (table 2). P. T. Waalkes of the National Heart Institute (personal communication) found very comparable serotonin and histamine production in the DON-S and RII lines. P815-DON-RI, on the other hand, produced only half as much histamine and about one-third as much serotinin. Such coincidental functional variation is to be expected between drug-resistant or any other mutants extracted from a heterogeneous parent tumor.

The outward identity of the three sublines of plasma-cell tumor 70429 (fig. 5C) and the absence of new chromosome types from their idiograms suggest true point mutations to A-methopterin resistance and dependence. Among the more than 40 mouse neoplasms analyzed in our laboratory, this is without doubt the most diploid tumor material. In its steep

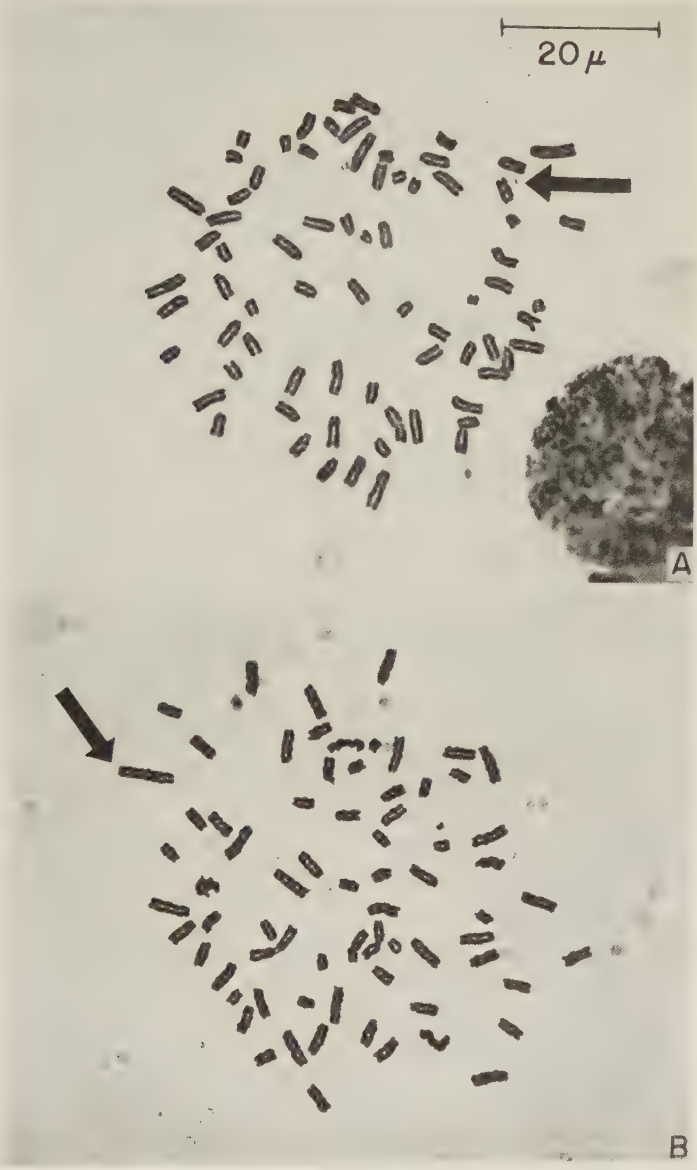

Fig. 4 A. Modal cell type of tumor E-NMF-S with 77 chromosomes, including two minutes. The smaller minute is indicated by arrow.

B. Modal cell type of subline E-NMF-R with 72 chromosomes. The extra-long marker (arrow) is present in 81% of the resistant cells but absent in all but 3% of the NMF-S cells. Two or three metacentrics may be seen below the center of the plate.

mode of over 80% diploid cells, it approaches Hungerford's data ('55) for the chromosome numbers of embryonic mouse cells.

Even in a length comparison with the mouse spermatogonial idiogram (fig. 6A), the plasma-cell tumor (fig. 6B) looks like a true diploid, whereas the neoplastic mast cells (fig. 6C, D) are pseudodiploids. They may be internally rearranged by translocations besides the obvious metacentric and extra-long marker chromosomes. Plotting of the logarithms of individual absolute chromosome lengths in serial sequence from 1 to 40 eliminates variables attributable to such differences as contraction stage and fixation and permits unbiased cell-to-cell comparison of relative chromosome lengths. Cryptostructural departures of tumors from the normal mouse idiogram (Levan, '56b) involving subtle rearrangements or losses of genic material became apparent when logarithms of length were plotted as in figure 7. Curve III, for example, which represents a stem-cell line of tumor P815-RI, differs in slope from the normal spermatogonial cell plotted in curve I, meaning that it contains both longer and shorter chromosomes.

The following conclusions may be drawn from the cytologic comparison of these tumor sublines (table 3).

1. Resistance phenomena occur readily in both diploid and heteroploid neoplasms.

2. In four of seven instances, the resistant or dependent lines were cytologically indistinguishable from the sensitive parent tumor; in three cases, resistance was accompanied by

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Fig. 5 A. Significant downward shift of the chromosome number in an NMF-R subline of the hypotetraploid Ehrlich ascites tumor. Each histogram is based on 100 exact counts.

B. Dissimilarity of the chromosome constitution in sublines DON-RI and DON-RII of mast-cell ascites tumor P815. Line DON-RII is indistinguishable from the sensitive parent line P815-S. Each histogram includes 100 exactly counted metaphases.

C. Similarity of chromosome number distribution in three sublines of the plasma-cell ascites tumor 70429. The histograms for 70429-S and AM-D are each based on 100 exactly counted metaphases; 50 metaphases were counted for the subline 70429-AM-R.

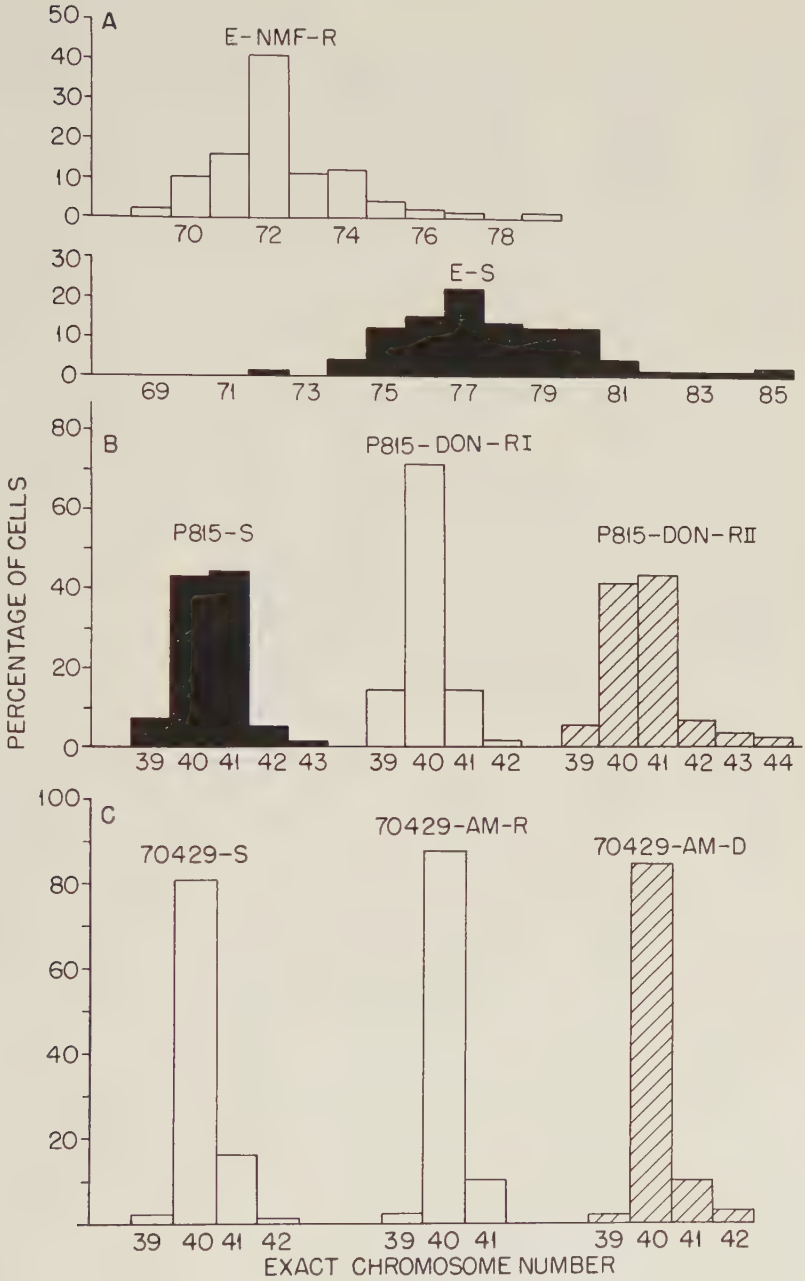
A
E-NMF-R
E-S
B
P815-DON-RI
P815-S
P815-DON-RII
C
70429-S
70429-AM-R
70429-AM-D
PERCENTAGE OF CELLS
EXACT CHROMOSOME NUMBER

Figure 5

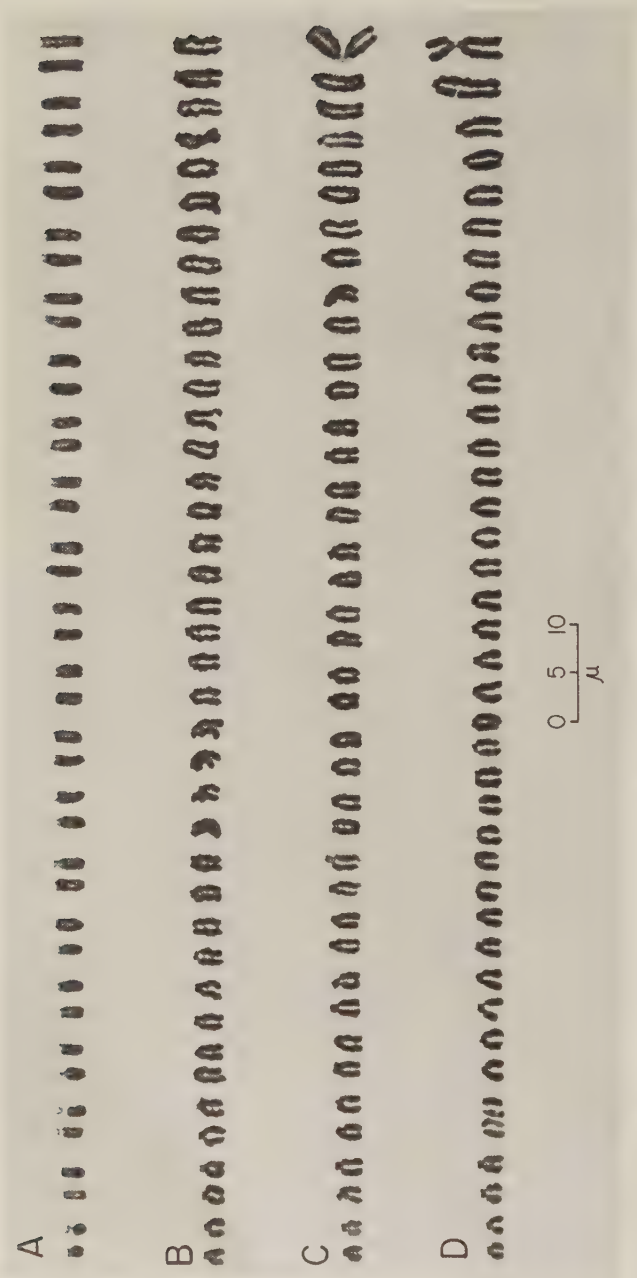

Fig. 6 A. The 40 chromosomes from a normal diploid spermatogonial cell of a newborn mouse (cut from a photograph by Hungerford, '56, and rearranged as pairs in order of size).

B. Idiogram from the diploid plasma-cell tumor metaphase (70429-AM-D) shown *in situ* in figure 2A.

C. Stem-line idiogram of the mast-cell tumor P815-DON-S (line A). Because of the generally poor spreading qualities and overlapping in this material, this is a composite idiogram; the large metacentric marker had to be borrowed from another cell.

D. The 40 stem-line chromosomes of the mast-cell tumor P815-DON-RI are taken from the metaphase pictured in figure 2F. The extra-long and unequal-armed metacentric (Nos. 39, 40) are obvious markers. Chromosome 31 has a secondary constriction and can be identified in many well-spread plates.

clear numerical and morphological departures from the corresponding sensitive idiograms. The inference of a causal relation between these chromosome phenomena and the mutation to resistance is uncertain, especially since a period of serial tumor transfers intervened between the origin of resistance and the cytologic study.

3. Although these findings do not contradict a strictly point-mutational concept of drug resistance, the gross nuclear innovations that are viable constitute another potential source of differences in chemotherapeutic response.

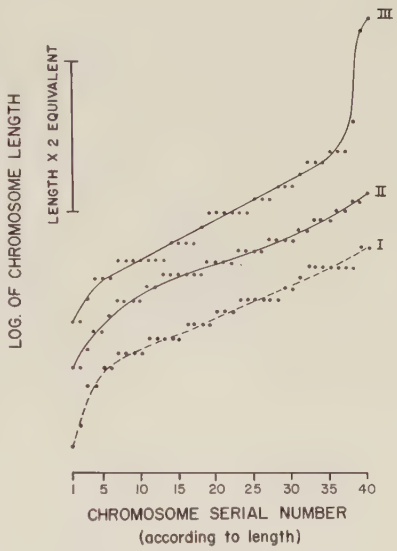

Fig. 7 Chromosome length measurements plotted on semilog paper and corresponding to three of the idiograms in figure 6 as follows: I = A (normal mouse spermatogonial cell); II = B (plasma-cell tumor 70429-AM-D); III = D (mast-cell tumor P815-RI). This manner of plotting reveals differences not only in length extremes, but also in the slope of length increment within each idiogram.

HETEROPLOIDY AND ANTIGENIC DIFFERENTIATION

Genetic control of histocompatibility among inbred mouse strains is well understood (Snell *et al.*, '53; Amos *et al.*, '55; Gorer, '56; Hoecker, '56). The isoantigens governing the fate of homografts are the only cell features measurable on the somatic level for which a background of precise Mendelian information is available. The strongest, experimentally most useful of these antigens are determined by the *H-2* system located on linkage group IX. From earlier transplantation data, *H-2* has been interpreted as a simple locus with a number of allelic forms. Now we know that it is a complex chromosomal region embracing many closely linked pseudoalleles, as well as true multiple alleles. Four instances of crossing

TABLE 3

Chromosomal features of several drug-sensitive, -resistant, -dependent mouse ascites tumors

TUMOR LINE	PLOIDY		MODAL CHROMOSOME NUMBER	CELLS WITH MARKER CHROMOSOMES			
	Near diploid	Near tetraploid (and a few octoploids)		Extra long	Metacentric		Minutes
					Unequal arms	Equal arms	
	%	%		%	%	%	%
Plasma-cell neoplasm							
70429–AM–S	98.8	1.2	40	—	—	—	—
70429–AM–D	98.7	1.3	40	—	—	—	—
70429–AM–R (129)	98.8	1.2	40	—	—	—	—
Lymph-node leukemia							
P288–AM–S	0	100	~ 80	—	—	—	Most cells
P288–AM–RII	0	100	~ 80	—	—	—	1 or 2
Mast-cell tumor							
P815–DON–S (line A)	99.2	0.9	40 and 41	—	—	35	—
P815–DON–RII (176)	98.1	1.9	40 and 41	—	—	43	—
P815–DON–RI (69)	97.1	2.9	40	100	93	—	—
Ehrlich ascites tumor							
E–NMF–S	0	100	77	3	38		61
E–NMF–R	0	100	72	81	70		13
Lymphosarcoma							
P1798–CORT-S	1.0	99.0	~ 80	—	—	—	Several
P1798–CORT–R	88.6	11.4	41	—	—	—	Occasional

over within the *H-2* complex are on record (Allen, '55; for other references, see Hauschka and Amos, '57).

Strain-specific mouse tumors have been the experimental tools in amassing these findings, because of their antigenic simplification, as compared with the much larger number of immunogenetic prerequisites of normal tissue. This simplification may proceed to the point of complete genetic indifference, at which point the tumor grows in any host strain. Searching for cytogenetic parameters of indiscriminate homotransplantability, we have established a functional relation between chromosome balance and antigenic differentiation (Hauschka and Levan, '53; Hauschka and Schultz, '54; Hauschka and Amos, '57), confirmed in other laboratories (Sachs and Gallily, '56; Mitchison, '56). Our experiments were based on a broad histologic spectrum of 25 mouse tumors with modal chromosome numbers in the diploid or tetraploid region. Systematic comparison of the graft specificity and the *H-2* antigens of this material showed an inverse relation between karyotypic balance and transplantation range. Tumors with diploid modes usually had strong *H-2* antigens and were not transplantable beyond limits determined by inheritance of these antigens. All tumors capable of progressive growth across isoantigenic barriers had rearranged, heteroploid chromosome complements. The latter were either decidedly hyperdiploid or, more often, near tetraploid. This karyotypic imbalance was usually, but not always, accompanied by a significant weakening of the tumor's capacity to absorb *H-2* hemagglutinins out of specific isoantisera.

Gorer and Mikulska's methods of antibody detection ('54) have enhanced the sensitivity of these test sera down to dilutions of 1:16,000. Conclusions regarding relative amounts of *H-2* substances on the surfaces of diploid and heteroploid tumor cells have, therefore, a reliable quantitative basis (Hoecker and Hauschka, '56; Amos, '56; Hauschka and Amos, '57). We may summarize our findings by emphasizing that significant weakening of the *H-2* antigens is the most frequent

effect of heteroploidy; complete disappearance of an *H-2* configuration from a tumor cell surface is quite rare, apparent isoantigenic strengthening has been observed in only one case among over 30 mouse tumors. Besides these quantitative changes associated with chromosomal imbalance, qualitatively new antigens of the X type have become apparent in near-diploid lymphomas (Gorer and Amos, '56; Amos and Day, '57). It is not at all surprising that antigenic differentiation is frequently abnormal in cells with aneuploid nuclei. Altered chromosome sets should be expected *a priori* to have a profound influence on so specific a configuration as an *H-2* substance.

Experiments show that even superficially diploid tumors are antigenic mosaics, in keeping with their karyotypic diversity. Kaziwara ('54) selected polyploid sublines by passing the predominantly hyperdiploid Ehrlich ascites through immunized Swiss mice. A similar immunoselection occurred in our diploid lymphosarcoma 6C3HED during serial passage in certain refractory genotypes toward tetraploidy, lower virulence, and weak antigenicity (Hauschka *et al.*, '56). The original diploid lymphoma was highly virulent and invasive; it was, and still is, compatible only with $H\text{-}2^k$ mice. From take ratios in backcross hybrids, it was apparent that this tumor differs from the DBA/2 strain by one factor, the difference being *H-2* antigens K versus D. In spite of this normally potent barrier, the lymphosarcoma was selected toward greater compatibility with the resistant hosts. The DBA/2-adapted subline had acquired a near-tetraploid chromosome constitution and greatly reduced virulence; its hemagglutinin-absorbing titers were feeble.

Immunological differences also exist between individual cells in tumors that are already polyploid. For example, the MC1M fibrosarcoma originating in C3H mice gave a strong K reaction, yet grew in (and killed) C57BL mice lacking this antigen. The ascites revealed an intense granulocytic reaction against the tumor, and the mouse sera had high titers of anti-K after small inocula. With large inocula, no granulocytic

reaction or antibodies were found (Amos, unpublished). These phenomena raise the question whether serologic results pertain to the cell population as a whole, or whether small residues of antigenic mutants can grow and kill the host despite a deceptive immune response.

Cytotoxic antisera, produced in C57BL mice and containing guinea pig complement and trypan blue (Gorer and O'Gorman, '56), showed that the MC1M fibrosarcoma is indeed a mixed population with respect to immunologic properties. The more-antigenic cells were lysed immediately; others disintegrated and stained slowly; 20–30% of the tumor cells did not take up the dye and remained intact (Amos, unpublished data).

The so-called universal tumors, like the polyploid Krebs-2 and Ehrlich ascites, seem to lack detectable *H-2* antigens. However, clones derived from single Ehrlich cells, and differing in their cytologic and functional properties (Hauschka, '53a; Hauschka and Levan, '58), have begun to reveal formerly hidden serologic specificities. The latter were encountered after tumor regression and subsequent immunity in mice of the inbred 129/Rr strain (table 4). None of these mice survived after inoculation with the hypertetraploid clone E1 (mode of 83 chromosomes). On the other hand, the seven hypotetraploid clones E2 through E8 (chromosome modes, 72 to 76) killed only about half the inoculated 129/Rr mice. After temporary tumor growth and regression, survivors remained tumor free and resisted lethal challenge with the corresponding clones. Cross-immunity between clones E2 and E4 was demonstrated, but there was only incomplete protection against the original stock tumor. Presumably, the latter contains some less antigenic variants, similar to the ancestral cell of clone E1.

The antisera produced in the 129/Rr mice against the Ehrlich ascites clones E2 and E4 had cytotoxic and hemagglutinating properties. When Amos tested them against red cells from 16 different mouse genotypes, they revealed the

TABLE 4

Antigens in certain clones of the Ehrlich ascites shown by regression and subsequent immunity in 129/Rr mice

MOUSE STRAIN	MICE SURVIVING AFTER INJECTION OF 1–20 MILLION TUMOR CELLS					
	Ehrlich stock		Hypertetraploid: clone E1		Seven hypotetraploids: clones E2 to E8	
	%	Survivors/ no. injected	%	Survivors/ no. injected	%	Survivors/ no. injected
HaICR Swiss	0	0/676	0	0/354	0	0/364
DBA/2	0	0/5	0	0/7	0	0/37
C3H/St	0	0/10	0	0/10	5	3/64
C57BL	15	2/13	0	0/10	3	1/30
129/Rr	7	2/29	0	0/11	46 [a]	52/114

[a] Twenty-three survivors, tumor free 2 months after regression of E5 and E8 were challenged with E stock (20×10^6 cells); 14 died but 9 were immune and remained tumor free. Thirteen tumor-free survivors after inoculation with E2 and E4 had both cytotoxic and isoagglutinating antibodies in their sera. They were completely immune to challenge with E2 cells (2×10^6), showing cross immunity between two hypotetraploid clones.

presence of three isoantibodies. The first of these is directed against an *H-2* antigen (probably M); the second also seems to be an *H-2* antibody formed against a new antigen in the *H-2* complex; the third has not yet been tested for linkage. Intact living cells of clone E2 absorbed these antibodies.

Antigenic overlapping with certain nuclear components of our Ehrlich source tumor is indicated by preliminary results of Amos and Hauschka with nuclear proteins prepared by Dr. J. S. Colter. The hemagglutinating properties of the 129-anti-E2 serum were removed by deoxynucleoproteins (DNP) but not by ribonucleoproteins. These results need further testing. If confirmed, they would agree with skin-graft data of Billingham, Brent, and Medawar ('56), showing that DNP and histocompatibility factors may have properties in common and can both provide antigenic stimuli in transplantation immunity. Where only the surfaces of uninjured cells come in contact with a refractory host, the immunogenetic character of the surface would be paramount in shaping antibodies.

A most unexpected result was the discovery of new *H-2* components in clonal cells from a nonspecific tumor. The ability of these aneuploid cells to immunize only about half the 129/Rr mice furthermore implies residual segregation of antigens in an inbred mouse strain. Pitfalls inherent in such experiments should be appreciated. Somatic variation in gene-determined antigenic traits, clearly subject to modification by gross chromosomal variability, must — for want of anything more stable — be investigated in host systems that are themselves suspect of germinal heterozygosis in corresponding antigens.

SUMMARY

Exact chromosome analyses of diverse mammalian tumors support the conclusion that most cancers are multiclonal mosaics of altered karyotypes. Tumor progression toward physiologic autonomy is facilitated by cytogenetic instability. Although we cannot ascribe altered cell functions to definite

chromosomal changes, some general consequences of heteroploidy for cell behavior are apparent.

1. With respect to growth potential, comparable diploid and polyploid tumor cells show no basic differences. They are able to synthesize the same protoplasmic mass within the life span of the host mouse. To attain this mass, the diploid tumor produces twice as many cells as the polyploid.

2. A relation may exist between the chromosome constitution of ascites tumors and susceptibility to oncolytic viruses. Certain neurotropic agents are oncolytic, and multiply by preference in the most aneuploid neoplastic cell populations.

3. Polyploid tumor cells usually do not contain balanced multiples of the haploid species genome, and may even be monosomic for whole chromosomes or regions. Therefore, they do not consistently show decreased radiation sensitivity such as occurs in some balanced polyploid yeasts and higher plants.

4. In a comparison of drug-sensitive tumors with their resistant or dependent sublines, several differences in chromosome number and structure were found. It cannot be determined whether these clear chromosome changes represent the cytogenetic basis for selection or are coincidental with true mutations to resistance and dependence. The uniform presence and stability of marker chromosomes in some resistant stem lines suggest that they carry genes essential for cell survival.

5. Certain changes in the isoantigens of tumor cells are correlated with over-all karyotypic balance. All the transplantable mouse tumors that can grow across isoantigenic barriers have rearranged heteroploid chromosome complements. Tumors with diploid stem lines regress in foreign strains of mice. Serologic evidence indicates weakening or disappearance of some, but not other, isoantigens in most cells with irregular chromosome sets. Some neoplastic cell populations (even among the nonspecific universal mouse tumors) are antigenic mosaics in keeping with their karyo-

typic diversity. They can be separated into pure clones with different immunologic properties demonstrable by protective immunization.

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CHROMOSOMAL CHANGES IN PRIMARY AND TRANSPLANTED RETICULAR NEOPLASMS OF THE MOUSE

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TWO FIGURES

INTRODUCTION

The idea that abnormal chromosome sets may have something to do with carcinogenesis is usually first attributed to Hansemann (1890). Boveri ('14) expressed the hypothesis in something approaching modern cytogenetic terms and Winge ('30) almost echoed Boveri some 20 years later. But the views of Boveri and Winge languished for lack of reliable information about chromosome number and form in neoplastic cells. The result was that the mounting knowledge of the irregularities of the mitotic process in cancerous tissue drew attention away from a possible underlying cytogenetic constancy, and it is only in recent years that attention has been focused once more on the chromosomes themselves. The reasons for this reawakening interest in the chromosomes were technical — the demonstration of the value to experimental oncology of ascites tumors by Yoshida ('56) and his followers in Japan and by the Kleins (Klein *et al.*, '50; Klein and Klein, '56) in Europe, and the use on these tumors of cytological techniques based on the squash method of Heitz ('36) by K. Bayreuther,

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T. S. Hauschka, P. C. Koller, A. Levan, S. Makino, J. H. Tjio, and others. Together, the new materials and techniques provided information of a standard hitherto unattainable. The main outcome was the discovery that most ascites tumors of rats and mice consisted of populations of mixed cell types that varied in their chromosome content about a modal set usually different in number, and often somewhat in form, from the basic diploid chromosome complement of the species. Since cells with the same modal set of chromosomes were perpetuated on transplantation, it was suggested that they were the "essential" or "stem-line" cells of the tumor and that the remaining cell types were variants that had arisen from the stem-line cells. The weight of evidence in favor of this view is now very strong (Levan, '56; Makino, '56).

It is no criticism of the valuable results from such work on ascites tumors to point out that in two respects they are not representative of neoplasms generally: they are largely free-cell populations (in which cell competition might be expected to be severe), and the great majority have been transplanted for many transfer generations. The results obtained may therefore not be wholly applicable to primary and "solid" tumors.

Some 2 years ago, a cytological technique developed for another purpose was found to give results often equal in quality to those obtainable with ascites cells when applied to a variety of primary neoplasms of the mouse, particularly those of the reticuloendothelial system. We therefore decided to conduct a survey of the chromosome cytology of a range of these primary tumors and their transplanted derivatives.

MATERIAL AND METHODS

Origin of the neoplasms and kinds studied. Most of the neoplasms examined arose in irradiated mice of the CBA strain (Mole, '58). Others appeared in irradiated C57BL and A mice, and some in unirradiated mice of the same three strains as well as in mice bearing the strain designation FAK

I.[2] The method of selection of tumors for examination was arbitrary. Although many different types were examined, only neoplasms arising in reticular tissue will be discussed here. To the unaided eye, these were readily divisible at autopsy into three types: (1) thymic leukemias with a large mediastinal mass but no obvious involvement outside the chest; (2) generalized leukemias with involvement of lymph nodes and spleen, and frequently of liver and other organs; and (3) more-focal reticulosarcomas usually appearing in older animals. The first two types would come within the group of lymphocytic leukemias of Dunn's classification ('54), and the third largely into her reticulum-cell sarcomas, class A. At sacrifice, records were taken of the gross pathological features, and specimens from organs with known or suspected involvement were fixed for histological examination. From many of them, pieces of involved spleen or lymph node were broken up in saline and then injected intraperitoneally or (more usually) subcutaneously into fresh young animals of the same strain. The number of cells injected was estimated to be in the range 10^6 to 10^7. Most of these transplanted neoplasms grew in their new hosts. As might have been expected, growth time varied considerably among different neoplasms, particularly in the early transplant generations. Although no deliberate attempts were made to convert any of these growths to the ascites form, in several instances an ascites tumor appeared after intraperitoneal injection.

Cytological technique. The animals were injected with a solution of Colcemid[3] 1½–2 hours before sacrifice. Small pieces of tissue from various organs — spleen, lymph nodes, thymus, sometimes liver, and tumor (from subcutaneous transfer) — were chopped up in hypotonic sodium citrate solution, fixed in acetic alcohol (1:3), and stained in the Feulgen

[2] FAK I strain: believed to be related to Furth's AK strain. Obtained from the Chester Beatty Research Institute, London. Believed to have gone from Furth to Englebreth Holm to Chester Beatty.

[3] Colcemid (Ciba): a derivative of colchicine with equally efficient action but greatly reduced toxicity.

reagent. The femoral bone marrow, when taken, was handled as a cell suspension. The technique has been described in detail elsewhere (Ford *et al.*, '56). Squash preparations were made, sometimes after long storage of the stained tissue in 45% acetic acid in a deep-freeze unit (ca. —14°C.). Most of the preparations were made permanent by the dry-ice method (Conger and Fairchild, '53).

Observation and recording. Cells in metaphase were selected for examination by systematic search under the low power of the microscope (7 × eyepieces and 10 × objective). The aim was to accept for study only cells that appeared to be intact (as judged by the distribution of the chromosomes) and in which the chromosomes were reasonably well dispersed, fixed, and stained. Once chosen, a cell was not subsequently rejected if one or more groups of chromosomes turned out to be difficult to interpret. The chromosomes were counted by visually dividing the cell into sectors, writing down the number in each sector, and then summing. In positions where alternative interpretations were possible, a decision was taken in favor of the more likely one before the final tally was made. In this way a series of "exact" counts was obtained, and also a series in which the counts were known to be possibly in error by one or sometimes two chromosomes. The quality of the preparations inevitably varied to some extent. This was reflected by the proportion of cells rejected and the ratio of the numbers of "exact" to uncertain counts recorded. The "exact" counts always predominated, however, sometimes to the virtual exclusion of uncertain counts, and since the interpretation of the results is unaffected, the two series are combined in the tables presented.

Cells are not infrequently ruptured during the making of squash preparations, and the acceptance for counting of only the cells that *appear to be intact* is no assurance that all *are* intact. Some of the counts, although exact, may still be erroneously low through loss of chromosomes during handling. Spurious increases of chromosome number are also not impossible. A single free chromosome, released by the break-

up of a cell during squashing, may come to lie under or over another apparently intact cell in metaphase. Some such instances have indeed been identified through distinguishing the adventitious chromosome from the remainder by differences in staining, degree of contraction, quality of fixation, or a small difference of focal level. Experience, however, suggests that it would be exceptional for such an event to happen and not be detectable. Another way in which a spuriously high count might come to be recorded would be through the precocious division of a centromere and the counting of both chromatids as separate chromosomes.

Terms and symbols. Since counts of chromosomes are subject to error, even in the best preparations, it will be useful to distinguish between the chromosome *number* of a cell and the chromosome *count*, which is an estimate of the chromosome number.

The characteristic abnormal chromosomes that give a striking individuality to some neoplasms will be referred to as *marker* chromosomes. The simplest of these are changed only in length. Symbols have been used to denote the approximate magnitude of the change when measured relative to the longest and shortest of the apparently normal chromosomes, respectively. Since there is no guarantee that any chromosome *is* normal, this procedure might seem to be hazardous. It has been satisfactory in practice, however, and will be used here. The symbols and their interpretation in terms of relative length are as follows:

l = longest "normal";	s = shortest "normal":
$l' \sim 1.20\, l$	$s' \sim 0.67\, s$
$l'' \sim 1.50\, l$	$s'' \sim 0.50\, s$
$l''' \sim 1.90\, l$	$s''' \sim 0.25\, s$

Two other types of change occur less frequently. In one type, translocation of a chromosome segment to the short arm of another chromosome has given rise to a metacentric chromosome. Since such elements are absent from the normal set of mouse chromosomes, they are particularly valuable as markers. The other less-frequent type has a prominent secon-

dary constriction. This character is not always expressed and is therefore a much less useful marker. Diagrams of the marker chromosomes characteristic of each of the neoplasms described in detail are given at the foot of the relevant tables, the marker chromosomes themselves in solid lines, and (for comparison) the longest and shortest apparently normal chromosomes in broken lines.

RESULTS

The chromosomes of over 60 reticular neoplasms of the mouse have now been examined in one or more tissues of the primary growths or of their transplants or both. The volume of data assembled is too great to be presented in full; instead, a series of selected examples will be given. Details of irradiation (where given) and of gross pathological and histological diagnoses for each of the original host mice concerned are set out in table 1. Descriptions of the individual neoplasms will be followed by a classification of all reticular neoplasms studied, according to their principal cytogenetic properties.

Chromosomes of normal reticular tissues. To assess the significance of the chromosomal conditions in neoplasms, it is obviously necessary to know the extent to which numerical and morphological variation of the chromosomes may occur in the corresponding normal tissues. Unfortunately, few counts have been made in tissues of normal untreated mice. Many more have been obtained in connection with studies of radiation chimeras (Ford *et al.*, '56, '57; Ford, Ilbery, and Loutit, '57). Those that refer to isologous or homologous (T6/+) cells dividing in an irradiated host are recorded in table 2, combined with the few from untreated animals. No morphologically changed chromosomes were observed in any of these cells, and since there is no reason to suppose that they would show less variability than cells from intact animals, the counts may be regarded as providing evidence of the chromosome numbers of cells in normal tissues.

TABLE 1

Six selected neoplasms: irradiation and pathological details

NEOPLASM NO.	MICE		TOTAL RADIATION DOSE [a] AND PERIOD COVERED		ANATOMICAL DIAGNOSIS	MICROSCOPICAL DIAGNOSIS
	Strain	Age				
		mo	*r*	*wk*		
1	CBA	19	1550	9	Generalized nonthymic leukemia	Lymphoid leukemia (typical)
23	CBA	11	250	2	Generalized leukemia (including thymus)	Lymphoid leukemia (typical)
26	CBA	27	1250	10	Mammary sarcoma; pulmonary adenoma	(Lymphoid leukemia)[b]
47	CBA	22	500	10	Subcutaneous reticulosarcoma with spleen involvement	Reticulum-cell leukemia
77	FAK 1	11	(Spontaneous)		Generalized leukemia (including thymus)	Lymphoid leukemia (primitive)
155	C57BL	12	1000	4	Generalized nonthymic leukemia	Lymphoid leukemia (primitive)

[a] All animals were females about 3 months old when irradiation commenced. The dose was delivered in several subacute fractions spread over the period indicated.

[b] Appeared after transplantation of mammary sarcoma (see text).

TABLE 2

Chromosome counts in tissues of normal untreated mice and of irradiated mice with reticular tissues regenerated from injected isologous or homologous cells

ORGAN OR TISSUE	NO. OF MICE	NUMBER OF CELLS CONTAINING THE INDICATED CHROMOSOME COUNTS												TOTAL CELLS
		≦30	31	32	33	34	35	36	37	38	39	40	41	
Bone marrow	45	8	3	2	1	4	4	7	6	20	45	545	1	646
Spleen	13	5	1	0	1	1	3	8	9	6	23	149	1	207
Lymphoid	5	0	0	0	0	1	0	1	2	5	3	42	0	54
Total	50	13	4	2	2	6	7	16	17	39	71	736	2	907

The one-sided distribution of the counts in table 2 is very striking. Ideally, a count of exactly 40 in every cell would have been expected if there is a constant somatic number. Errors of interpretation superimposed upon constancy of number could lead to an approximately symmetrical distribution of counts. The skewness of the observed distribution might therefore merely reflect the inclusion of cells from which chromosomes had been lost as a result of damage. True deviation from the diploid number might arise in two ways: (1) by elimination of chromosomes through noncongression at metaphase or by lagging at anaphase or (2) through nondisjunction. If sufficiently frequent, elimination alone could give rise to a distribution of numbers like the one observed. It would lead to the appearance of micronuclei, however, and although no special search was made for them, none were seen in the several hundred preparations examined. Nondisjunction alone would be expected to give rise to a symmetrical distribution of numbers around a mode of 40, or perhaps to a distribution with some positive skewness since, in general, duplication is less deleterious than deficiency. The expected distribution is therefore clearly not in accord with the observed one. The rare occurrence of elimination and nondisjunction is not excluded, of course, but the considerations just given form the basis for our belief that, in normal cells of the four tissues examined, true deviation from the standard diploid number of 40 is exceptional, if it occurs at all.

Our belief in the numerical constancy of the chromosomes in somatic cells is strengthened by some unpublished results obtained by G. B. Sharman. They point to the conclusion that, after irradiation, spermatogonia that have lost chromosome segments do not reenter mitosis (and probably die). The inference is that if loss of a portion of the chromosome is sufficient to prevent a cell from reappearing in mitosis, loss of a whole chromosome can hardly be less deleterious. Sharman's observations were made on males of the small marsupial species *Potorous tridactylus*, which have 13 large and

well-differentiated chromosomes in their somatic cells (five autosomal pairs plus X, Y_1, and Y_2). Conditions for cytological observation were therefore particularly favorable.

Transplanted leukemia N23. No material for cytological examination was taken from the original host, and the first observations were made on preparations of spleen from the first transplant generation. The modal cell type of this leukemia proved to be remarkably constant, the mode of 41 chromosomes having persisted from the first transplant generation to the twenty-first (table 3). A single *s′* chromosome was present throughout this period (see fig. 1A). The relatively low numerical variability is noteworthy; it may be contrasted with the much wider spread of the counts obtained on material of leukemia N1.

Transplanted leukemia N1. Cytologically, this leukemia is characterized by a modal chromosome number that shifted during the early transplant generations and then became stable, by a wide range of variation about the mode, and by a highly specific set of five marker chromosomes (table 4 and fig. 1B). The latter consist of two *s″*, one *s′*, and one *l′* together with a chromosome of medium length that has a clear distal secondary constriction. The two *s″* and the one *l′* chromosomes were recognized in all the tissues of the primary growth examined, but the other two markers were not detected until the second transplant generation, and even there they were not recorded regularly. It is obvious that the more exceptional or abnormal a chromosome is the more easily will it be recognized, and the occasional omission in the records of some of the set of five markers is not evidence that they were not there: they were regularly identified in the good cells. Even in the poorer cells, the two *s″* chromosomes served as reliable markers, and it was therefore a simple matter to distinguish between normal cells (40 chromosomes; no markers) and leukemic cells (usually more than 40 chromosomes and two to five markers). It is noteworthy that after cells of N1 had been stored in a tumor bank for several months and

TABLE 3

N23 Transplanted leukemia from an irradiated CBA host

TRANSPLANT GENERATION	NUMBER OF CELLS CONTAINING THE INDICATED CHROMOSOME COUNTS					TOTAL CELLS
	≤ 40	40	41	42	43	
1			5			5
2	1	2	3	2		8
3		8	30	6	1	45
4	1	4	24	2		31
11	1	4	35	1		41
21	1	3	14	1		19
Total	4	21	111	12	1	149

Marker

TABLE 4

N1 transplanted leukemia from an irradiated CBA host

TRANSPLANT GENERATION	NUMBER OF CELLS CONTAINING THE INDICATED CHROMOSOME COUNTS							TOTAL CELLS
	≤ 39	40	41	42	43	44	45	
Primary	7	41	10	3	1	1		63
1a				2	5	2	1	10
2a				14	7	2		27
1b	1	2	10	3	1			17
2b	1	6	20	6				33
9b	1	1	9	7	4			22
10b	1	2	18	15	2	1	1	40
Total	11	52	71	50	20	6	2	212

Markers

Figure 1

A Cell with 41 chromosomes from mesenteric lymph node. N23, eleventh transplant generation. One marker chromosome.

B Cell with 42 chromosomes from spleen. N1, tenth transplant generation. Five marker chromosomes.

C Cell with 40 chromosomes from subcutaneous tumor. N26, fifth transplant generation. Six marker chromosomes.

D Normal cell with 40 chromosomes from spleen. N26, eighth transplant generation. All 4000×.

then reestablished in live mice, all five markers were still present in the thirty-first passage generation.

Transplanted leukemia N26. An irradiated CBA mouse developed a mammary tumor and was sacrificed for cytological examination. At the same time, pieces of the tumor were transplanted into fresh CBA mice. At autopsy of the original animal, a small lung nodule was discovered, and part of it was also taken for cytological examination. Although no satisfactory mitoses were found in the mammary tumor and the lung nodule was nearly as bad, one cell was recorded as containing 48 chromosomes including two metacentrics and six small chromosomes. By the third transfer of the mammary tumor, however, a generalized lymphoid leukemia had developed that proved to be the most remarkable one cytologically we have yet encountered. It had a modal number of 41 and a set of six marker chromosomes even more striking than those of N1. There were two large and distinguishable metacentric chromosomes, three *s″* (all distinguishable by size), and one *l″* (fig. 1C). Unlike the markers of N1, all these are easily detected and, in the great majority of cells examined, all six were recorded as being present. The similarity of this chromosome set to the chromosomes found in the cell from the lung specimen is far too great for it to be attributed to chance. A reasonable explanation is that, in addition to the two tumors already mentioned, the original animal carried a leukemia in an early phase and that leukemic cells had already become widely disseminated, being associated with the mammary tumor and also present in lung.

Several separate transplantation lines of this leukemia were established, one of which developed a modal number of 43 chromosomes with the standard set of six markers plus a fourth *s″*. In another line, there was a progressive shift from a mode at 41 to a new mode at 40 during transplant generations four to nine, but without alteration of the basic set of six markers (table 5). In animals carrying N26, normal cells with 40 unchanged chromosomes can be distinguished from

the leukemic cells with even greater assurance than in N1. The normal cells recorded have therefore been excluded from table 5, which accordingly refers to the leukemic cell population only. In this and other leukemias where it is possible to distinguish them, cells with normal chromosomes are especially prevalent in spleen and bone marrow (as might be expected), but may also be present in any other organ or tissue invaded by leukemic cells. A normal cell from the spleen of an animal carrying N26 leukemia is shown in figure 1D.

The marker chromosomes of N26 provide an excellent opportunity for examining the constancy of the stem-line set. Disregarding the variations in total chromosome number, 203 cells had the full set of six (or seven) markers, nine cells lacked one marker, and in nine other cells one marker was duplicated. The latter are worth examining further: in one cell the larger metacentric chromosome was duplicated, in four cells the smaller metacentric, and in four cells one of the small chromosomes. Since the cells concerned were scattered in preparations of six different tissues or organs of 13 different animals in three transfer generations, they provide no evidence for the existence of cell clones additional to the modal type. On the contrary, the distribution of additional marker chromosomes is much as would be expected if all the numerical variation had arisen by random nondisjunction. The shift from a 41-chromosome mode to a 40-chromosome mode, however, was presumably through the appearance of a new, more-successful, clonal type.

Transplanted reticulum-cell sarcoma N47. The cytological interest of this example lies in the occurrence of a bimodal distribution of chromosome numbers in the tumor and spleen of the primary host, which was maintained (at least in spleen) through seven generations of transplantation. Table 6 shows clear modes at 41 and 43. When material from another anatomical site (axillary lymph node) was examined in the seventh transplant generation, however, a single very sharp mode at 43 was found. In this leukemia there must therefore

TABLE 5

N26 transplanted leukemia from an irradiated CBA host

TRANSPLANT GENERATION	NUMBER OF CELLS CONTAINING THE INDICATED CHROMOSOME COUNTS					TOTAL CELLS
	≤ 39	40	41	42	43	
4	4	12	71	5	1	93
7	1	5	9			15
8		19	18	1	2	40
9	2	11				13
Total	7	47	98	6	3	161

Markers

TABLE 6

N47 transplanted leukemia from an irradiated CBA host

TRANSPLANT GENERATION	ORGAN OR TISSUE	NUMBER OF CELLS CONTAINING THE INDICATED CHROMOSOME COUNTS							TOTAL CELLS
		39	40	41	42	43	44	45	
Primary	Spleen	4	8	12	6	20	4	1	55
5	Spleen and ascites	4	7	27	17	36	9	7	107
7	Spleen	1	2	13	2	25	3	1	47
	Axillary lymph node	1		2	3	37	4		47
	Total	10	17	54	28	118	20	9	256

Markers — none.

have been two successful clonal types. The strong representation of only one of them in the axillary lymph node could be attributed either to an adaptive preference for that particular anatomical environment (which we consider to be the more likely) or to chance early seeding of the site by a 43-chromosome leukemic cell.

Spontaneous primary leukemia N77. This case was chosen partly to include a spontaneous leukemia and partly because it offers a striking instance of differences between organs or tissues in their content of leukemic cells (table 7). As in N47, there was an over-all bimodal distribution of chromosome numbers, this time with peaks at 42 and 44. The 44-chromosome type predominates in bone marrow and thymus, the 42-chromosome type in the axillary lymph node. The spleen and perhaps the mesenteric node have both types. Two small chromosomes are regularly present in cells of both the 42- and 44-chromosome types.

Since such a mouse would contain vast numbers of leukemic cells, it seems much more likely that the differences between organs are associated with adaptation of the cells to particular sites rather than chance seeding. But the point could be regarded as proved only if effectively the same distribution of chromosome numbers within and between organs were to be found again after transplantation.

Primary leukemia N155. The cytological material of this generalized leukemia was technically excellent, particularly that from the spleen, in which all the cells counted contained either 40 or 41 chromosomes, except three that were probably damaged (table 8). In the absence of marker chromosomes, we assumed that the 40-chromosome cells were normal and the 41-chromosome cells neoplastic. This interpretation was strengthened when preparations from the mesenteric and paratracheal lymph nodes were scored and no cells with 40 chromosomes were found. In the bone marrow (which was examined later), however, four cells were found that contained an exceptionally small chromosome (s'''), and one cell with

TABLE 7

N77 Spontaneous leukemia in original host (FAK I)

ORGAN OR TISSUE	NUMBER OF CELLS CONTAINING THE INDICATED CHROMOSOME COUNTS								TOTAL CELLS
	≦39	40	41	42	43	44	45	46	
Bone marrow	7	4		4	4	29		1	49
Spleen	1	1		21	3	12		1	39
Thymus	1			1	1	13	1		17
Mesenteric node				4	2	8	1	1	16
Axillary node		1	1	29	1	5		1	38
Total	9	6	1	59	11	67	2	4	159

Markers

TABLE 8

N155 Primary leukemia in an irradiated C57BL mouse

ORGAN OR TISSUE	NUMBER OF CELLS CONTAINING THE INDICATED CHROMOSOME COUNTS								TOTAL CELLS
	36	37	38	39	40	41	42	43	
Bone marrow		1	1	4	38	6			50
Spleen	1	2			30	67			100
Mesenteric node				1		23	1		25
Paratracheal node				1		13	10	1	25
Total	1	3	1	6	68	109	11	1	200

Markers — none.

an exactly matching *s'''* plus a long chromosome (*l''*). The implication of successive chromosomal change is strong and since 40 chromosomes were counted in four of these cells (and 39 in the fifth), the presumption is that at least some of the apparently normal 40-chromosome cells of both marrow and spleen may have been neoplastic.

There is a possible alternative explanation. Clones of cells with rearranged chromosomes are now known to arise frequently in heavily irradiated mice treated with homologous or heterologous bone marrow and in which host-type hemopoietic tissue has subsequently regenerated (Ford, Ilbery, and Loutit, '57; Barnes *et al.*, '58). These changes, however, are invariably *balanced;* that is, there is no evidence of loss or gain of chromosome material and the chromosome number remains constantly 40. On the other hand, the chromosomal changes in reticular neoplasms are frequently *unbalanced,* as we have shown. But for the suggestion of successive rearrangement (which is not found in the irradiated, marrow-injected mice), it would have been reasonable to suppose that the 40-chromosome *s'''* cells were members of a clone of rearranged normal cells, and to dismiss this possibility on the evidence of one cell is not justified.

Cytogenetic classification of reticular neoplasms. We have found it convenient to classify the neoplasms of the mouse according to the following cytogenetic properties: (1) modal chromosome number, (2) presence or absence of numerical variability about the mode, and (3) presence or absence of marker chromosomes. These properties have been used to classify 60 independently arising reticular neoplasms (see table 9). Classification was based on a minimum of ten cells examined, an average of 30 to 40, and a maximum (in several of the transplanted growths) of over 300. In the few reticular neoplasms from which material was taken for study but that are excluded from the table, the preparations were technically unsatisfactory.

Ninety per cent of the growths arose in irradiated animals, and most of the remainder were spontaneous leukemias that developed principally in FAK I mice. No clear distinction by cytogenetic features is apparent between the spontaneous and the induced types. In most instances, diagnosis of cytogenetic type was based on the primary growth alone; in many, on primary growth and transplant; and in some, on early transplants alone.

TABLE 9

Reticular neoplasms of the mouse: cytological characteristics

CYTOLOGICAL CHARACTERISTIC	TYPE OF NEOPLASM					
	1	2	3	4	5	6
Modal number	40	40	40	40 +	40 +	80 ±
Variability	—	+	+	+	+	±
Marker chromosomes	—	—	+	—	+	±
Number of neoplasms observed (total, 60)	9	14	8	13	15	1

It is noteworthy that only nine of 60 reticular neoplasms were indistinguishable from normal tissue (as far as the examination went) and that 23 of 60 had new marker chromosomes. The other point worth notice is the rarity of polyploid types. (It should be added that the highest modal number so far recorded in the hyperdiploid range is 46). In addition to the one frank tetraploid neoplasm, two others had a pronounced (but not dominant) near-tetraploid component. These two cases were classified according to the characteristics of the hyperdiploid component. Apart from these three cases only occasional tetraploid cells were seen. Certainly, no tetraploid subpopulation became established in any of the transplanted lines.

Origin of chromosomal variation. It is reasonable to suppose that variation in chromosome number about a diploid or hyperdiploid mode is attributable to nondisjunction. Evidence that this is so is given by the cells of N26, in which one or another of the marker chromosomes was duplicated. Several

instances of the occurrence of new morphologically changed chromosomes were noted in the various transplanted lines, but these were usually confined to single cells. In N1, a minor subpopulation of cells containing a new small metacentric chromosome was present in one transplant generation but was not detected again. The subline of N26 with a mode of 43 chromosomes and an additional small chromosome has already been mentioned: in this case, however, it is possible that the new type arose after nondisjunction of one of the original small chromosomes rather than a new rearrangement.

Many instances of primary structural changes have been seen in the course of the work, including examples of both chromosome- and chromatid-type rearrangements. These observations were very scattered and are insufficient for the formulation even of very rough rules of occurrence — whether, for instance, they are more plentiful in one transplanted neoplastic line than in another or in one tissue than another (both of which are suspected). Nevertheless, the impression persists that they are usually more frequent in primary than in transplanted growths. Although, in most of the recorded examples, the changes were present singly, several cells were seen in which many independent primary structural changes had occurred.

DISCUSSION

Validity of the observations. Before attempting to interpret the observations, we must inquire whether they can be accepted unreservedly. Three questions arise: Are the counts correct? Does the selection of cells for counting introduce a bias? Are the cells actually in mitosis a fair sample of the whole neoplasm? Although some samples may have been unrepresentative and some of the counts wrong, the very fact that in all our transplanted neoplasms the same modal chromosome number and the same set of marker chromosomes were maintained in successive transfer generations, except for occasional minor shifts, is sufficient indication of the general accuracy and representative character of the results. It also

gives confidence in the reliability of the results obtained on primary neoplasms not checked by transplantation.

The individuality of reticular neoplasms. We have shown that the reticular neoplasms of the mouse, whether spontaneous or radiation induced, frequently differ from normal tissue and from one another in three cytogenetic features: (1) the modal chromosome number, (2) the extent of variation about the mode, and (3) the presence of distinctive marker chromosomes. Taken together, these may confer on the neoplasms a pronounced individuality. This reaches its extreme in the unique characteristics of N1 and N26. Our series includes types with fewer or less-distinctive marker chromosomes, types with no marker chromosome but a hyperdiploid mode, others with a mode at 40 and some variability, and a final diploid group with (apparently) no greater variability than the corresponding normal tissue. It is not yet clear whether we have been dealing with two distinct neoplastic types — those in which chromosomal variations have occurred and those in which they have not — or whether all should be regarded as members of a continuous series. Although nine representatives of the invariant diploid group were recorded (table 9), none were studied extensively, and it is possible that they would have revealed greater variability than normal tissue if more extensive counts had been made. As it happens, none of our transplanted lines had a fixed diploid set of chromosomes, though this may only reflect chance selection of primary growths for transplantation.

The chromosomes of the mouse show very little differentiation; they are all acrocentric and differ in relative length only through a range of about 1–2.5. We have evidence (from radiation-induced translocations) that many rearrangements would go unsuspected in an examination of somatic chromosomes alone. It is therefore not only possible but also very probable that many chromosomal changes were not detected in the neoplasms examined. If these could have been identified, many of our series probably would have shown more striking evidence of their individuality.

In view of the insensitivity of mouse chromosomes for revealing chromosome rearrangements, we have attempted to induce neoplastic changes in the Chinese hamster by irradiation. This species has 22 pairs of reasonably well-differentiated chromosomes in its somatic cells. The only case to appear as yet was diagnosed as a lymphatic leukemia, and it is not yet fully analyzed. In the bone marrow, however, 89 of the mitotic cells were of one highly distinctive type, all containing 23 chromosomes that displayed no less than nine good marker features. Thirty-seven of the cells had entirely normal chromosome sets, 25 cells were obviously related to the main neoplastic type, most of them having one chromosome more or less than the standard set of 23. The relationships of the seven remaining cells require further study; they may represent stages in the development of the standard 23-chromosome type. This case gives support to our belief that the chromosomal changes that we have been able to demonstrate directly in murine reticular neoplasms represent only a part of the changes that have actually occurred, and that the true extent of their cytogenetic individuality is greater than it appears.

The mere demonstration of numerical and structural changes in the chromosomes, however extensive and however typical of the individual neoplasm, is insufficient for proof of effective genetic change. All that can be rigorously inferred is duplication of some centromeres, but the circumstantial evidence that distinct chromosomal types do in fact reflect genetic differences is strong.

The stem-line hypothesis and cell selection. The regularity with which the same modal set of chromosomes was observed over many transplant generations is evidence not only of the consistency of the observations and of the individuality of the neoplasms but also of the continuity of the modal subpopulation of cells. This conclusion is fully supported by observations on all the transplanted reticular neoplasms we have studied.

Factors that might have favored changes were minimal since transplantation was always carried out into animals of the same sex and the same highly inbred strain to maintain the physiological environment provided by successive hosts as invariant as possible.

The concept of stem-line cells derived from the observations on ascites tumors is therefore shown to be applicable to another large class of neoplasms, and is demonstrated with particular clarity by the continuity of the highly specific sets of marker chromosomes of N1 and N26. The implication of this hypothesis is that the modal (stem-line) type is the most "successful" neoplastic cell type; that variants, though presumably arising continuously, are less "successful" and are as continuously eliminated (otherwise there would be a progressive rise in the over-all cytogenetic variability of the neoplasm). In other words, the neoplastic cells vary in their "fitness" to survive and multiply, and selection eliminates the less fit.

The stem-line hypothesis was originally proposed to account for the observations on ascites tumors. These tumors were obtained by serial transplantation over many generations, and it was always conceivable that selection during transplantation was responsible for the cytogenetic characters observed in the later generations. There were no observations on the primary tumors by which this could be checked. It therefore seems important that the same kind of observable changes are to be found in primary neoplasms and that the cytogenetic characters are largely stable during the initial stages of serial transplantation, i.e., for 10 to 20 generations. Thus the stem-line hypothesis seems to be just as applicable to the primary neoplasms as to the end result of selection after serial transplantation for many generations.

The validity of the stem-line hypothesis is not affected by the coexistence of two (or even more) separate clonal types within a neoplastic cell population as, for instance, in N47 (table 6) since these may have balanced advantages such as adaptation to different anatomical sites or growth phases. Nor

is it affected by the ability of one type to be slowly replaced by another, as in N26 (table 5). Indeed, the last is one of the best illustrations that selection really is operative; a more successful new type has taken the place of the old.

Since the kind of selection that is seen to occur is between separate chromosomal types, we may infer that chromosomal differences are in some way concerned with differences of individual cell physiology that render one type more successful and another less so. The obvious interpretation is that the chromosomal types are the morphological indicators of distinct cell genotypes.

New cytogenetic variation. We now know enough of the normal constancy of chromosome form and the process of spontaneous and induced chromosome rearrangement to be quite sure that morphologically new chromosomes can arise only as a result of chromosome breakage and reunion. Levan in particular (e.g., '56) has stressed the role of structural changes in the evolution of the chromosome complements of ascites tumors, especially those with near-triploid or near-tetraploid modes.

Among the different primary structural changes seen were two examples of a type of chromatid aberration that might well be of considerable importance, not in connection with the origin of new morphological chromosome types (because the amount of chromatin transfer would nearly always be too small), but rather for the finer cytogenetic changes that must be assumed to take place from time to time. The nature and assumed origin of the two aberrations are illustrated in figure 2, which is based on extensive experience of this kind of rearrangement gained during studies of chromosome breakage in root tips of *Vicia faba.* They represent one type of the class of chromatid interchanges (Revell, '55) and are particularly frequent after exposure of the roots to nitrogen mustard solution. The interpretation given is confirmed by Slizynska ('57), who found the expected reverse repeats (and also direct repeats) in the salivary gland chromosomes of

Drosophila larvae after chemical treatment of their male parents. The potential importance of the particular type illustrated lies in the production of daughter nuclei, one of which would be duplicated and the other deficient for the same small chromosome segment. Theoretically, there is no lower limit to the size of the affected region. We envisage a continuous series terminating in an exactly balanced exchange of segments between sister chromatids.

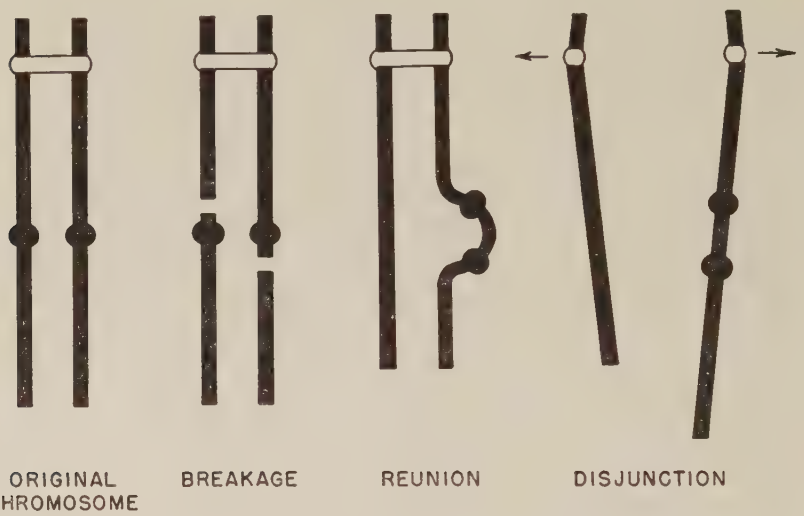

Fig. 2 Diagram to show the origin of duplication and deficiency from chromatid intrachange.

Straightforward deletion, of course, can arise from chromosome-type breakage and reunion (i.e., change involving effectively single strands); for duplication of a segment to occur the two-stranded state is necessary. Other mechanisms besides the one discussed are possible, but all others would involve a minimum of three breaks.

So far as we are aware, there is no known exception to the rule that all agents that cause chromosome breakage are also mutagenic. It is therefore probable that the extent of the genotypic variation that occurs in these neoplastic cells runs the whole course from duplication and deficiency of whole

chromosomes through changes involving major and minor chromosome segments to "gene mutation," and the frequency of all is much higher than in the corresponding normal tissue.

Significance of the chromosomal changes in reticular neoplasms. The observations made show that there is variation in the number and form of the chromosomes in reticular neoplasms of the mouse, and reasons have been given for believing that more-extensive genetic variation occurs than can be revealed by the methods used. The persistence of a modal stem line combined with variation shows that the variant cell types have different selective values, and there must be regular elimination of the less-efficient types with, as was observed, occasional emergence of one that is more successful. The stability of the cytogenetic individuality of each tumor line from the time it was first examined in the primary host leaves the fundamental problem still open, the problem of whether the neoplastic process began in a catastrophic event involving major rearrangments of genetic material or in a gradual accumulation of a series of changes, each small in itself. Once a variational mechanism came into being, selection would be inevitable and by the time a primary tumor had reached the size of those examined here, adaptation to the primary host might very well be complete — hence the relatively minor shifts during subsequent transplantation into secondary hosts, providing very nearly the same physiological environment as the primary host. The essential problem is how the mechanism for variation was initiated.

In the preceding paragraph, the cytogenetic individuality of neoplasms is assumed to be of causal significance. If the observed changes in chromosomal pattern were merely epiphenomena, a wider range of variability would have been expected than was observed: a persistent stem line and a persistent set of marker chromosomes would have been a most unlikely, not a regular, occurrence. Another view would be to consider the chromosome pattern as visible evidence of consolidation by genetic change of a modification originally

imposed by other means, a process similar to that which Waddington ('57) considered an evolutionary factor in sexually reproducing organisms and called "genetic assimilation." Nevertheless, there must be a real difference of this "normal" process from the "abnormal" process of carcinogenesis since, in genetic assimilation, the ability to survive in the original environment is lost; in neoplasms, it is retained. The most important observations for the immediate future must be on the earliest stages of the neoplastic process that it may be possible to examine. Here the cytological technique available and the consistency with which reticular neoplasms can be produced by irradiation show real promise of giving worthwhile results.

SUMMARY

1. There is very little (if any) aneuploid variation in chromosome number in the reticular tissues (bone marrow, spleen, lymph node, and thymus) of normal mice. No alterations of chromosome form have been observed in these normal tissues.

2. In a relatively small proportion of primary reticular neoplasms of the mouse, there is no direct evidence that the chromosomes differ from those observed in the corresponding normal tissues.

3. The great majority of these neoplasms, whether spontaneous or radiation induced, show a real variation in chromosome number that is usually confined to an over-all range of five or less. They also usually differ from the normal in that (1) the mode about which variation occurs is often in the range 41 to 45 and (2) one to six distinctive new (i.e., morphologically changed) *marker* chromosomes may be present as a regular and characteristic feature of the chromosome set. In many of the neoplasms of this majority group, the combination of a particular modal number with a group of new marker chromosomes gives each a distinct cytogenetic individuality.

4. On continued transplantation, the distinctive chromosomal properties of the primary growth are maintained, ex-

cept that there may be occasional shifts in modal number and even more occasional incorporation in the modal set of new chromosome types.

5. There is indirect evidence that nondisjunction is frequent in cells of primary and transplanted reticular neoplasms, and direct evidence that primary structural change also occurs. Reasons are given for believing that the observable gross changes of chromosome number and form represent only a small part of the total cytogenetic variation in these neoplastic cells.

6. The interpretation placed on the observations reported is that the chromosomal changes are indicative of genetic change, and most of the reticular neoplasms examined therefore consist of populations of cells of many different genotypes, each population being not only different from a normal cell population but also in many instances clearly different from other neoplastic cell populations. Furthermore, we believe that, in transplanted lines, new cytogenetic variation is continuously arising and is as continuously eliminated. Finally, we interpret this process of elimination to be not random but selective, so providing the basis for both the normal maintenance of the characteristic modal set of chromosomes on transplantation and its occasional replacement by another, related modal set.

7. The relation of the observed changes to the neoplastic process is not yet clear. The changes may be an integral part of the causal complex, in which case two possibilities are envisaged: (1) a catastrophic event involving major rearrangements of the genetic material or (2) a gradual (selective) accumulation of a series of changes, each perhaps small in itself. If selective and cumulative, the process may be either primary or possibly commence secondarily and represent a consolidation by genetic change of a modification originally imposed by other means.

ACKNOWLEDGMENTS

We wish to thank our colleague Dr. K. W. Jones for permission to incorporate some of his data on N23, and also to express our appreciation of the efficient technical assistance given by Mr. G. Breckon, Miss P. A. Moore, and Miss S. R. Wakefield.

OPEN DISCUSSION

(Papers by T. S. Hauschka and C. E. Ford *et al.*)

YERGANIAN [4]: The inference that I gather from statements made by Drs. Hauschka and Ford that the mouse has cytologic advantages over the Chinese hamster may, in the absence of comparative data, signify that the mouse is better than the Chinese hamster, rat, monkey, and human karyotypes.

The Chinese hamster, with only 11 pairs of readily distinguished chromosome forms, permits us to (1) relate each type of chromosome in determining the degree of aneuploidy, (2) trace the formation of new chromosomes among spontaneous and induced tumors, as well as during the course of heteroploid alteration of normal cells grown *in vitro* and, consequently, (3) judge the survival capacity of different types of aberrations in proliferating tissues. A number of 20-methylcholanthrene-induced fibrosarcomas have exhibited a variety of karyologic states in the absence of new chromosome forms. For example, tumor CH-5MC possessed the normal disomic or classic diploid pattern but failed to transplant readily beyond the twelfth passage; whereas tumor CH-38MC exhibited perpetual nondisjunction among the near-diploid cells; consequently, a high degree of nullisomaty was witnessed until around the forty-fifth passage. At this time, several hypotetraploid sublines, each possessing new chromosome forms, appeared.

We have noted that some cells having the so-called diploid number of chromosomes are actually aneuploids. About one-third of the aneuploids, or 10% of the near-diploid population of normal bone marrow and regenerating liver cells, are made

[4] George Yerganian, Children's Cancer Research Foundation, Boston.

up of these quasidiploids. Individuality of the chromosome forms once again clarified this heretofore unrecognized entity among mammals (Tonomura and Yerganian, '56, '57). Functional or viable aneuploids and quasidiploids will serve as fruitful sources for clonal propagation of monosomics and trisomics of normal and malignant cells *in vitro*.

The first readily transplantable adenocarcinoma of this species has now been established *in vivo* and *in vitro* (Yerganian and Gagnon, '58). Although this particular tumor has a hypotriploid modal frequency of 28 chromosomes, a single trisomic count for any one of the chromosome forms has yet to be encountered. Instead, normal or modified chromosomes, or both, are either disomic or tetrasomic. Thus chromosome number or modal frequency can fail to reflect the true nature and range of ploidy.

So it seems that a heteromorphic karyotype enables us to see things that are considered quite impossible in the mouse.

Hauschka: Of course chromosomes of the Chinese hamster are fewer in number and perhaps better distinguishable than the less well-differentiated idiogram of the mouse, but in the mouse we have the advantage of a great deal of genetic information from years of strain development and inbreeding, which we do not have in the hamster. In particular the mouse isoantigen permits correlations between physiological and chromosomal properties of somatic cells.

Ford: The uniformity of mouse chromosomes has one great advantage — metacentric chromosomes and major changes in chromosome length are very easily identified. The diversity of the Chinese hamster chromosomes has a different advantage — that changes of a magnitude quite undetectable in the mouse can be recognized. This was so in the one lymphoid leukemia of the hamster we examined cytologically. The chromosomes had all the advantages of detail that Dr. Yerganian mentioned, but each cell took a very long time to analyze. The two species with their different proper-

ties should be regarded as complementary, not alternative or rival.

KOLLER[5]: Dr. Ford counted the chromosomes in over 900 cells of the mouse and almost everyone of these cells had 40 chromosomes, which is the normal diploid number. This finding is of special significance in view of the many recently published reports describing great variations in chromosome numbers of cells maintained in tissue culture and of the tendency shown by the authors to question seriously the constancy of the normal diploid chromosome number in the somatic cells of the individual.

Although cell variation occurs rarely in normal tissues, it is common in tumors. This has been known for many years and was suspected of being one of the causes responsible for the unpredictable behavior of tumors during therapy. By using excellent cytological techniques and serological methods, Dr. Hauschka obtained overwhelming evidence of the wide range and diverse type of variation that can exist in malignant cell population of ascites tumors. He stated that the variation occurs during the progression of tumors. Perhaps the term progression is misleading. It implies the development of the tumor toward a more-complex state than it had at the start, when actually it is undergoing changes leading toward anaplasia, a state that represents morphological as well as physiological simplification as regards the organization and behavior of the tumor, and is usually associated with loss of many cellular properties.

Dr. Hauschka showed that in some tumors correlation exists between chromosome numbers' antigenicity and drug resistance; in others no such clear relation could be demonstrated. Ford reported that gross deviation from the diploid chromosome number is not a universal phenomenon, consequently it is not a necessary condition of malignant change. These findings indicate that the process of malignant transformation is complex; in some instances it might be attributable to genetic

[5] P. C. Koller, Chester Beatty Research Institute.

change in the chromosomes only; in others, the initial change might be in the cytoplasm. And we should consider the possibility that both nuclear and cytoplasmic components may be involved in the initiation of a tumor. How varied may be the initial process is indicated by the origin and behavior of endocrine-dependent and virus-induced tumors. Intensive study of the diverse variations occuring in tumors, by methods described by Dr. Hauschka, can be expected to throw new light on the initiation of malignant change and perhaps on the process of differentiation itself.

KLEIN [6]: May I comment for just one moment on the term tumor progression. This is just a term, and it has no meaning unless defined. Foulds defined it in the technical sense. His definition includes all kinds of stable, heritable, and irreversible changes in one or more characteristics of an established tumor. This is quite a useful concept. Foulds studied the occurrence of such changes of various types and formulated some empirical rules that are not without interest in this discussion.

He found that progression is random and unpredictable. We do not know when a given tumor is going to occur and which characteristic it will involve. If there are several different primary tumors in the same host, such as two independent mammary carcinomas or two stilbestrol-induced testis tumors, progression occurs independently in each of them, showing that it is caused by a change in the tumor cells and not in the host. Furthermore, progression with regard to different characteristics occurs independently; e.g., a tumor may undergo progression from dependence on a certain hormone to independence. It may also change in growth rate, degree of differentiation, drug sensitivity, invasiveness, or metastasizability. These changes are not necessarily interrelated and may occur quite independently from each other.

When different characteristics of a series of primary tumors of the same type are compared, the tumors are often found to represent different stages in a progressional series

[6] George Klein, Karolinska Institutet.

not related to their size. Each tumor can progress further, either in the primary host or after serial transfers. There is no end point to progression; each given state can be replaced by still more-autonomous states.

In the thyrotropic pituitary tumors studied by Furth, the first change is an experimental disturbance of the normal hormonal balance of the host by radiothyroidectomy. The pituitary gland responds by enlarging, often very considerably. Here the primary change is not in the cells of the pituitary but in the host, as Furth has pointed out. An enlarged pituitary of this kind transplanted further to a thyroidectomized host of the same strain will usually grow progressively. Thus it behaves like a malignant tumor but is different because its growth can be checked by administration of thyroid hormone.

This is only the first step, however. If this dependent (or conditioned) tumor is kept by serial transfer in thyroidectomized mice, sooner or later it will change and become able to grow in normal mice of the same strain, too. This new form will be independent of the original hormonal imbalance. This change is a new and different one that has occurred in the tumor cells themselves. There are many other analogous cases.

The great range of variability in tumor cell populations is not surprising. I do not think it is very useful to discuss what is a primary and what is a secondary change, and I do not think we ought to look for simple relations between cause and effect. Perhaps we should think in terms of multiple correlations between different factors and should regard tumor development not as one or two cellular changes but as an evolutionary process. The story of any given evolutionary process is probably quite different in detail from every other process of the same nature; so is probably the developmental history of every single tumor. We bring them all together into one group and try to fit them to simple concepts, perhaps merely because of the common end result, which happens to be a disease.

BRILES [7]: The generally broad protective action engendered by immunizing against a tumor differing from the host in a single *H-2* allele could be fully explicable on the assumption that the serological and genetic nature of the *H-2* system parallels that of the extensive multiple allelic blood group series in cattle and chickens. In these species, the cross-reactivity spectrum of antibodies formed in a single antiserum against the antigenic product of any one allele is so broad that some degree of reaction will occur between the antiserum and antigens produced by most other alleles of the same system, except, of course, the recipient type. Thus the broad protection afforded by one *H-2* type against others is wholly compatible with the simpler hypothesis of multiple allelism at the *H-2* locus. That is, each *H-2* allele is producing a large macromolecule with a high degree of chemical similarity to those produced by the other *H-2* alleles, and the cross reactivity is largely dependent on general similarity in structure rather than on production of two or more chemically distinct antigens by each allele or chromosome block.

Second, I should like to emphasize the probable genetic significance of Dr. Hauschka's conclusions regarding the antigenic phenomena encountered in clones extracted from a particular *H-2* type. These are the results one would expect when testing mutant types of a particular blood group system with antisera previously prepared against other antigens of the series. Thus (1) antibodies in a particular serum would likely still be cross reactive with new mutants at the *H-2* locus, accounting for the infrequent loss of an antigen; (2) most mutants would be expected to produce antigens showing less cross reactivity than the homologous antigen producing frequent reduction in reactivity; (3) by the same token, only rarely would the mutant type be expected to show stronger reaction than the homologous antigen (a stronger reaction for heterologous antigens is occasionally observed in blood groups systems in chickens); and (4) mutant types would

[7] W. E. Briles, DeKalb Agricultural Association, Inc.

be expected to cross-react with numerous antisera against which the original antigen would be negative, accounting for the appearance of new antigens. As far as I am aware, these most interesting data presented by Dr. Hauschka are the first to have a direct bearing on mutations occurring at a blood group locus. The antigens occurring in extracted clones should serve as excellent material for the study of the serological and genetic nature of the *H-2* system.

FORD: I think I can summarize by saying that in both primary and transplanted tumors there is very extensive genetic variation. This is expressed in several different ways and provides the basis for the tumor progression of which Dr. Klein spoke. I regard the observable chromosomal changes as a pale reflection of the totality of these changes.

The problem is just when this variational system arises, whether it is secondary and superimposed or whether it is there from the beginning; whether it follows the primary neoplastic event (if this is definable) or whether it is in any sense causal. This is perhaps an unanswerable question: in any case it must be left for the future.

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A GENETIC CHANGE IN A TISSUE CULTURE LINE OF NEOPLASTIC CELLS [1]

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FOUR FIGURES

Most of the work I shall discuss was done in collaboration with Dr. R. Dulbecco. This work centers around a study of the origin and nature of certain polio virus-resistant cell variants obtained in a tissue culture line of human neoplastic cells, the so-called strain HeLa. My reason for choosing this study as the main subject of this paper is twofold: I hope that it will illustrate some of the problems that confront workers dealing with genetic changes in populations of animal cells grown in tissue culture, and, at the same time, give a fair picture of the methods of tissue culture that are at the moment available for the solution of these problems.

Strain HeLa was derived by Gey and coworkers ('52) in 1951 from a tissue culture explant of a human epitheloid carcinoma of the cervix; the strain has since been maintained continuously in tissue culture by serial passages. The chromosome constitution of strain HeLa is heteroploid; the chromosome numbers most frequently found range from about 65 to 90. Since the diploid chromosome number in man is 46, these numbers comprise both hypotriploid and hypotetraploid chromosome constitutions. A certain fraction of the cell population always shows higher degrees of polyploidization. The possible relation between this heteroploidy and our findings will be discussed in greater detail later.

[1] Aided by a grant from the National Foundation for Infantile Paralysis.

If we expose a culture of HeLa cells for a limited time to an amount of polio virus that allows a fraction of the cells to survive and repeat this procedure several times, we finally obtain a cell population that differs from the original HeLa cell population in both its morphology and its resistance to the virus (Vogt and Dulbecco, '58). As will become clearer later, we shall from now on call a cell virus resistant if it survives a short exposure to a standard amount of virus. It is, however, to be understood that such resistant cells are nevertheless susceptible to the virus if exposed either for a longer time or to larger amounts of the virus.

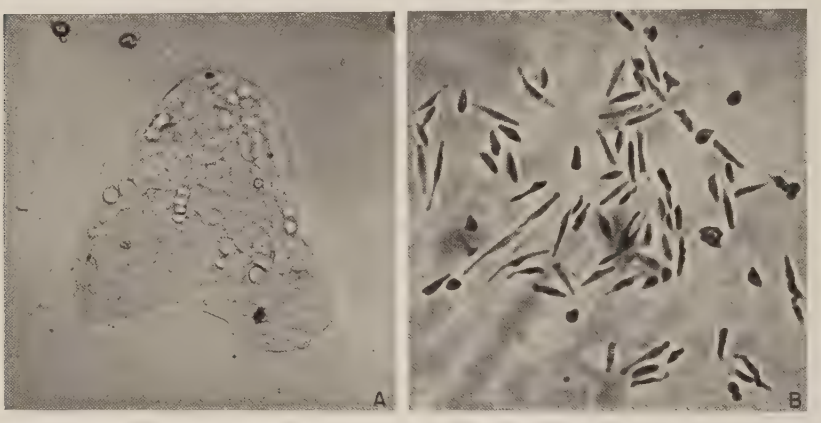

Fig. 1 A, Colonial morphology of standard S3 cells. Clonal derivative St1. B, Colonial morphology of resistant fusiform (F) cells. Clonal derivative F8.

Figure 1 illustrates the different morphology of the resistant cell population obtained after five successive short exposures to polio virus. Whereas the greater majority of the cells from a standard HeLa cell culture are polygonal in shape and form tight compact colonies (fig. 1A) in single-cell platings, the cells from our resistant culture are — in the presence of human or monkey serum — fusiform and have a tendency to migrate from one another, so that their colonies assume a loose texture (fig. 1B). The changed cell morphology and the increased virus resistance of our resistant culture

have now been maintained in the absence of virus for more than a year, which corresponds to more than 350 cell generations. Both traits may therefore be considered as genetic or hereditary.

The question arises whether HeLa cell variants with an increased resistance to polio virus occur spontaneously, i.e., independently of the interaction with the virus. That hereditary changes to drug resistance occur spontaneously in a line of transplantable mouse leukemia was first shown by Law ('52) by means of Luria and Delbrück's fluctuation test ('43). The use of the same test in our system was made difficult by the fact, already mentioned, that the resistant HeLa cell variants are nevertheless susceptible to the virus.

It was, however, possible to use an even more direct approach, based on the different morphology of the resistant variants. Single-cell platings from HeLa cell populations that have not been in contact with polio virus contain, at a relatively low frequency, clones with the fusiform morphology of the resistant variants. A clone of this fusiform type was isolated and found to be virus resistant. The genetic change to increased virus resistance occurred thus in this case spontaneously, i.e., independently of the virus. It may be of interest to mention that Burnet used, in 1929, a similar method to prove the spontaneous origin of mutations to bacteriophage resistance by picking colonies of rough morphology from a plating of a smooth strain and showing that these rough variants were resistant to phage.

I shall now describe in more detail the method used in this work to measure the virus resistance. Since an infection with polio virus leads to the death of the host cells, the proportion of cells that survive after exposure for a constant period to a virus preparation of determined titer was taken as a measure of the virus susceptibility of a given cell line. We determined the number of surviving cells by plating appropriate dilutions of the infected cell suspensions onto several petri dishes containing a medium supporting cell growth and, at the same

time, containing antibody to neutralize virus released by the infected cells. After an incubation period of 4 days, the cell colonies that had developed in each petri dish were counted by means of an inverted microscope. The method is an application of the cloning technique developed by T. T. Puck. By exposing aliquots of a given cell suspension to virus preparations of different titers and determining the surviving cell fraction for each virus multiplicity, we obtained cell survival curves as a function of the virus multiplicity. Such an experiment is illustrated in figure 2A, which shows the survival curves of two different cell populations. The lower curve

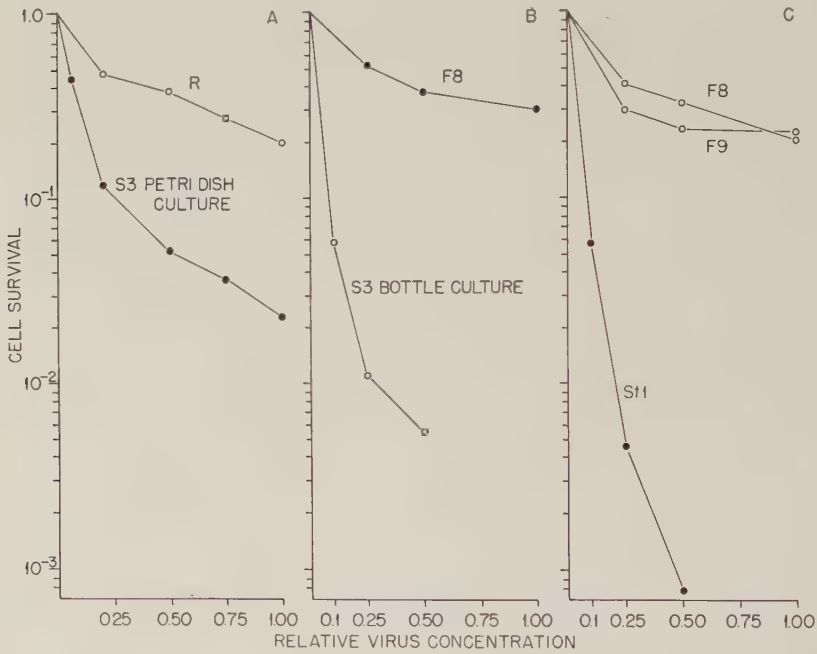

Fig. 2 A, Cell survival curves as a function of the virus multiplicity. The highest virus multiplicity corresponds to approximately five adsorbed plaque-forming units per cell. R, resistant cell population. B, Cell survival curves as a function of the virus multiplicity. F8, clonal derivative of the resistant cell population. C, Cell survival curves as a function of the virus multiplicity. F8, clonal derivative of the resistant cell population; F9, spontaneous fusiform clone from an S3 population, selected in the absence of virus; St1, clonal derivative of an S3 population and representing the standard S3 cell type.

represents the standard line of HeLa cells used in this work; the line was cloned in Dr. Puck's laboratory 2 years ago and has been designated as S3 (Puck and Fisher, '56). The upper curve represents the resistant (R) cell population I described, which was obtained from a standard S3 culture after five successive short exposures to polio virus. It can be seen that, for the same multiplicity of infection, the proportion of cells that survive as colony formers is significantly higher in the resistant than in the S3 population. The change in the slope of the survival curves shows, in addition, that both populations contain cells of different resistance. The cells showing the highest resistance (given by the final slope) have a probability of survival 15 times as great as that of the most sensitive cells contained in the S3 culture. The highly resistant cells comprise about 50% of the R population and about 10% of the S3 population. The curves also show that cells of high sensitivity are either absent or greatly reduced in number in the R population. It may be mentioned that the proportion of highly resistant cells in the S3 population was of the same order of magnitude as the proportion of fusiform cells present in a parallel noninfected culture.

Figure 2B shows the cell survival curves obtained from two other cell populations: the lower curve represents a line of S3 cells grown in bottles for more than a year; the upper curve represents a cloned cell line (F8) derived from the R population. Whereas the survival curve of the cloned F8 line is very similar to that of the original R population (fig. 2A), the S3 population derived from the bottle culture shows a greater sensitivity to the virus than the S3 population represented in figure 2A. In addition, only about 2% of the population derived from the bottle culture shows a higher resistance to the virus. Here, the proportion of fusiform cells in a parallel noninfected culture averaged 1%.

Figure 2C shows the survival curves of two additional cloned cell populations. The cells of the line, St1, correspond to the majority cell type of a standard S3 culture, and are

characterized by a polygonal shape and tight colonial morphology, as illustrated in figure 1A. Cells of this line, 2 months after isolation of the single cell, show a high susceptibility to the virus, as may be seen from the steep slope of the survival curve. Cells of higher virus resistance are either absent or are present at such low frequencies as to escape detection. Parallel with this finding is the fact that, over a culturing period of 4 months, we could not find clones of fusiform morphology in this line, although repeated single-cell platings were made at various time intervals. Line F9 was derived from the spontaneous fusiform clone, mentioned before, that was isolated in the absence of virus from a single-cell plating of the S3 bottle culture. The virus resistance of this fusiform cell clone is very similar to the virus resistance of the F8 line that was isolated from the resistant population obtained after repeated exposures to polio virus.

A rough estimate of the frequency with which resistant F cells are produced by standard S3 cells can be obtained by the uncorrected[2] formula for the mutation rate, derived by Luria and Delbrück ('43). According to this formula, the mutation rate, α, is given by the equation: $\alpha = (r/N)/g$, where g is the number of generations since the isolation of the single cell, and r/N the proportion of resistant variants after g generations. To estimate the proportion of resistant variants, we extrapolated the final slopes of the survival curves of the two S3 populations (shown in fig. 2A, B) to the origin. Its average is 6%. Assuming that these two cell lines had undergone 710 cell generations during the 2-year cultivation period since their derivation from a single cell, the value $0.06/710 = 8 \times 10^{-5}$ is obtained for α. If this estimate of 8×10^{-5} for the rate of production of resistant cells by standard S3 cells is correct, we would expect cloned S3 lines to contain an average of 3% resistant cells after a 1-year cul-

[2] It seems justifiable to use the uncorrected formula of Luria and Delbrück, which takes into account *all* cell generations, since enough F cells should already have been present at the first transfer (about 100 per bottle or petri dish) as to be carried over to the inoculum for the second transfer.

turing period. Tests for this prediction are planned for the near future.

The next question to ask is: What is the nature of the genetic changes that cause both the increased virus resistance and the aberrant morphology of the described HeLa cell variants? I have mentioned that the chromosome constitution of strain HeLa is heteroploid and may vary greatly from cell to cell. The possibility that the chromosome constitution might have some relation to the new characters of the variants was therefore investigated. Since this study was started only recently, the conclusions from the results so far available can be only tentative.

All chromosome counts were made on cell lines that were cloned recently and can be considered as derived with certainty from a single cell.

The method used for the single-cell isolation may be briefly mentioned. It is a slight modification of the technique described by Lwoff *et al.* ('55) for study of the virus release from single animal cells. The method consists in distributing individual cells from a trypsinized cell suspension in small drops of medium submersed in a large volume of paraffin oil, the purpose of the paraffin oil being to minimize evaporation of the droplets. The presence of only one cell in each droplet can be checked at high power with an inverted microscope. The method also prevents contamination of the outgrowing cell clone by cells from neighboring clones.

The results of the chromosome counts from three lines cloned by the method just described are summarized in table 1 and figure 3. These are the same cell lines, St1, F8, and F9, for which the cell survival curves at different virus multiplicities were shown in figure 2C. The figure shows the counts of approximately 50 mitotic figures of each of the three lines. Counts from mitotic figures with higher degrees of polyploidization are not included. As may be seen, the chromosome numbers most frequently found for line St1 lie between 78 and 80, for line F8 between 70 and 72, for line F9 around 76. All

TABLE 1

Chromosome counts from three clonal derivatives of HeLa S3

CELL LINE	CHROMOSOME NUMBER																TOTAL NO. FOR EACH CELL LINE	TIME AFTER ISOLATION
	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82		
																		mo
St1												10	14	5	1	1	31	3
											2	14	12	1			29	4
Total											2	24	26	6	1	1	60	
F8		1	3	13	1	4	2	1									25	6
	2		2	14	2	5	1	1									27	9
Total	2	1	5	27	3	9	3	2									52	
F9								2	1	15	11	2			2		33	1
									3	10	2	4	1				22	2
Total								2	4	27	13	6	1		2		55	

three lines differ thus from one another in their chromosome modes. Gross structural chromosomal differences between the three lines have not yet been detected. Figure 4 illustrates a representative chromosome figure of the lines St1 and F8.

The latest chromosome counts, not included in figure 3, show that the St1 line has maintained its chromosome mode over a culturing period of more than 6 months (or more than 180 cell generations), and the F8 line for more than 10 months (or more than 300 cell generations). The difference in the chromosome mode of the three cloned lines and the constancy

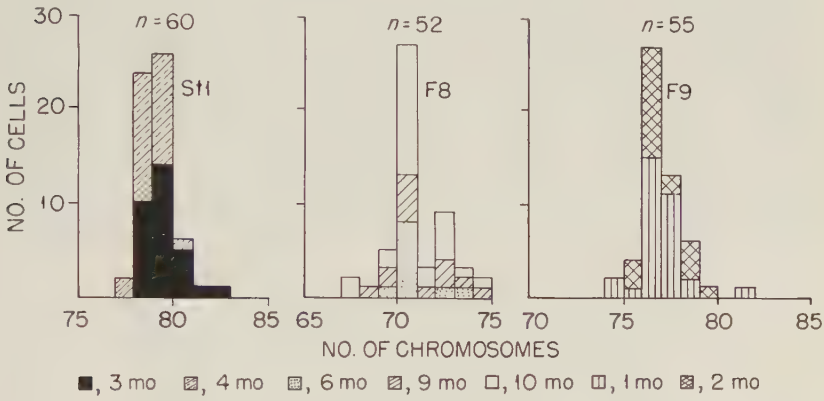

Fig. 3 Chromosome counts from the three clonal derivatives of S3. The different shades of the columns indicate the times (mo) since the isolation of the cell lines at which the counts were made.

with which this difference is maintained over a relatively long culturing period may therefore suggest that the genetic changes underlying the increased virus resistance and the aberrant morphology of the F8 and F9 variants are chromosomal. The difference between the chromosome modes of the F8 and F9 lines may furthermore indicate that two different events caused the two chromosome changes, which would suggest an independent origin of the two variants.

Having found that different cloned lines of HeLa cells differ in their chromosome numbers, we may finally ask: How frequently do such changes in chromosome numbers occur in

strain HeLa? The chromosome counts illustrated in figure 3 give us a partial answer to this question. As shown for line F9, chromosome numbers deviating significantly from the general

Fig. 4 Representative chromosome figures: A, from line St1 (78 chromosomes); B, from line F8 (70 chromosomes).

mode of the line were found as early as 1 month (or about 30 cell generations) after the single cell had been isolated. The same situation was found for cell lines F8 and St1; the counts from these earlier passages are not included in the figure.

The finding of atypical chromosome numbers in each of the three cell lines as early as 1 month after their isolation (earlier data are not available) demonstrates the extraordinarily high frequency with which numerical changes in the chromosomal constitution occur spontaneously in this tissue culture line of a highly malignant tumor. On the other hand, since the general chromosome mode of each of these three lines has kept constant over a period of several months, we have to assume that most of these observed numerical chromosome changes are either cell lethal or associated with a great selective disadvantage.

In summary, I may say that the results obtained so far are, at most, only suggestive of a relation between the increased virus resistance of certain HeLa cell variants and the observed chromosomal changes in these variants. We hope that a study of additional virus-resistant variants and preferably of variants derived directly from the cloned St1 line whose chromosome mode is known to have remained constant for several months will throw more light on this problem. In addition, a detailed comparative analysis of the chromosomal idiograms of the different cloned lines of HeLa becomes imperative if we want to bring definite evidence of the relation between the observed genetic and the observed chromosomal differences.

It seems of great interest to mention in this connection that Chu and Giles ('58) published chromosome counts from five mutant clonal derivatives of strain HeLa isolated in Dr. Puck's laboratory (Puck and Fisher, '56; Puck *et al.*, '57). All five clonal derivatives differ from one another and from regular strain HeLa in their biochemical growth requirements, and four of the five lines differ in their chromosome constitutions.

The idea that numerical and structural chromosome changes form the basis for the genetic variability of neoplastic cells has been held for many years by workers studying tumor cell populations *in vivo*. Extensive additional evidence has been given by Hauschka and by Klein (this symposium). It is, on the other hand, a known fact that many established tissue culture lines derived from normal tissues have acquired properties during the cultivation *in vitro* that often render them indistinguishable from tissue culture lines of neoplastic origin, both with respect to their growth properties and to their morphological appearance in *in vitro* culture. Since these "transformed" tissue culture lines are characterized by heteroploid chromosome sets (Hsu and Moorhead, '57; Berman *et al.*, '57; Westwood *et al.*, '57; Zitcer and Dunnebacke, '57, and others), it may be expected that some of the nutritional variants described in such lines (Chang, '57; Swim and Parker, '57) are also chromosomal variants. This expectation seems to be borne out by recent unpublished evidence of Dr. E. Chu, according to which a cloned line from Earle's strain L of mouse fibroblasts and a nutritional variant (McQuilkin *et al.*, '57) derived from this clone differ in their chromosome modes.

OPEN DISCUSSION

AUERBACH [3]: Is there any specific evidence that makes it likely that the chromosomal aberrations in permanent cell lines are the cause rather than the effect of the changed metabolism?

VOGT: I do not know of any evidence but I would favor the latter possibility, since the ordinary tissue culture medium appears not to allow easy growth of diploid cells.

LEDERBERG [4]: What is the dispersion of the chromosome modes of completely unselected clones of HeLa strains, clones isolated at random without regard to observed phenotypic differences?

[3] Charlotte Auerbach, Oak Ridge National Laboratory; on leave from University of Edinburgh.

[4] Joshua Lederberg, University of Wisconsin.

VOGT: I cloned ten unselected S3 cell lines for this purpose, but I have not yet made the chromosome analysis.

LURIA[5]: What is the possible role of the cloning process itself? The unusual and rather lonesome condition of a single mammalian cell that has to start to form a clone might produce an unbalance, which, in turn, might select or force some chromosomal abnormalities. Is there any evidence for differences in chromosomal numbers between cells cloned by themselves and cells cloned on the feeder layer?

VOGT: Such a comparative study has not yet been made. I think, however, that under the special conditions of these cloning experiments there would not have been a significant selective disadvantage, because the isolated cells showed a latent period and an exponential growth rate similar to that of cells in mass culture.

CAVALLI-SFORZA[6]: Is it easy to get rid of the virus when you select virus-resistant cells?

VOGT: Yes. I should tell a long story but I will make it very short. The resistant culture obtained after five successive exposures to polio virus was decontaminated after five successive treatments with antiserum. Cells from this resistant culture could be decontaminated immediately, however, if grown from single cells in microdrops. This difference in rate of decontamination is readily explained by the fact that reinfection occurs in the mass culture and is excluded in microdrops.

YERGANIAN[7]: Have you tried the virogenic potential of the virus-resistant line as compared with the usual susceptible HeLa?

VOGT: Resistant cells in which infection is successful have the same latent period and virus yield per cell as susceptible cells.

CAVALLI-SFORZA: I think of K12; of course, the difference is very great. Your lines seem to decrease in chromosome

[5] S. E. Luria, University of Illinois.

[6] L. L. Cavalli-Sforza, University of Pavia.

[7] George Yerganian, Harvard Medical School.

number when they have been made resistant. Could that mean that you are selecting for particular chromosomes?

LURIA: The question that cannot be answered is the following: Is it possible that the resistant cells may come from a presumably heterozygous condition and resistance is recessive to sensitivity?

VOGT: First, the resistance in K12 is caused by lack of absorption; we have here a different type of resistance. Second, the comparative analysis of the idiograms was planned to show whether one certain chromosomal class is systematically lost in all the resistant variants.

MERCHANT [8]: Is there 100% correlation between the virus resistance and the clonal morphology of the cell?

VOGT: Unfortunately I have only the two cases described; these show a 100% correlation.

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[8] D. J. Merchant, University of Michigan.

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GROWTH AND GENETICS OF SOMATIC MAMMALIAN CELLS *IN VITRO*[1]

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SEVEN FIGURES

Studies of genetic and biochemical processes in microorganisms have made enormous progress in the last two decades, largely because of the availability of simple techniques for carrying out the following operations: quantitative plating of cells under conditions that permit each cell to grow into a macroscopic colony; ready isolation of clonal stocks, and therefore also mutant clones whose divergent properties serve as genetic markers; and the growing of such cells in a chemically defined medium so that the metabolic differences among nutritionally divergent mutants could be delineated. The experiments here described were undertaken for the purpose of developing similar methodologies for studying genetic and biochemical relations in somatic mammalian cells.

Growth of large inocula of mammalian cells in tissue culture has long been a routine operation; Sanford *et al.* ('46) first demonstrated the growth of occasional single cells from selected strains into clonal populations. In a study of the factors controlling the reproduction of mammalian cells *in vitro*, we found that quantitative growth of single, isolated cells into readily identifiable colonies could be achieved simply and regularly (Puck *et al.*, '56; Marcus *et al.*, '56; Puck, Cieciura, and Fisher, '57). The measures necessary to accomplish this involved only minor differences from standard tissue culture procedures — differences in concentration and

[1] Contribution No. 75 from the Department of Biophysics, Florence R. Sabin Laboratories, University of Colorado Medical Center.

time of action of the agent used to produce monodisperse cell suspensions, in composition of the nutrient medium, and in regulation of the incubation conditions. By careful attention to such details, however, it was shown that single cells could be grown with efficiencies approaching 100% with the same regularity and almost the same ease as is routinely done in bacteriological laboratories (fig. 1).

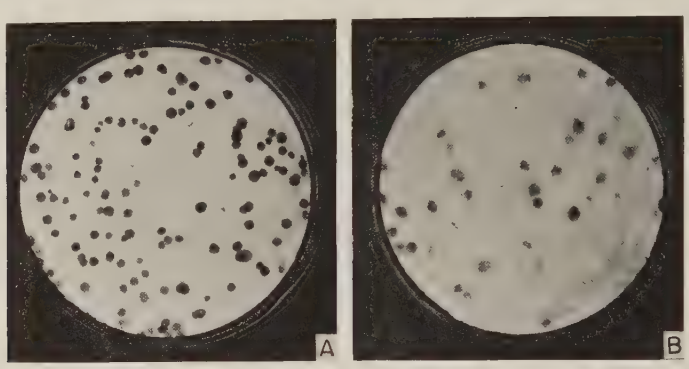

Fig. 1 A, Colonies developing from single cells of the S3 stock of the HeLa culture that originated in a human carcinoma of the cervix (Gey *et al.*, '52). (¾ ×)

B, Fibroblast-like colonies from the plating of single cells from normal human spleen. (¾ ×)

These procedures have been demonstrated to be equally applicable to cells from diverse organs like skin, bone marrow, liver, lung, spleen, ovary, and testis in man, the mouse, rabbit, chick, and Chinese hamster. Single cells from malignant as well as normal tissue, from both young and adult individuals and with euploid as well as aneuploid karyotypes responded similarly. It may be concluded that many of the cells of warm-blooded animals retain the capacity to multiply in isolation like independent microorganisms if they are furnished with the proper physical and chemical environment.

A variety of arrangements, different only in detail, can be used for the growth of these single cells, and these have made possible slightly different kinds of procedures for clone isolation. The single cells may be plated directly in a liquid medium in a petri dish to whose bottom they rapidly attach,

producing isolated colonies (fig. 1). Agar added to the medium (as in bacteriological procedures) ensures more stringently that cells from one colony do not contaminate another colony or set up satellite colonies. The agar renders the medium semisolid 3 hours after the initial plating, when the cells have become attached to the glass (Puck *et al.*, '56). Alternatively, the single cells can be plated into medium already containing agar, in which case they grow as spheres instead of as disc-like monolayers. Each such sphere represents a clone and can be readily "fished out" of the agar medium with a sterile wire loop. The most convenient method for clone isolation in our experience is to place over the colony grown on glass, a small metal cylinder whose bottom has been coated with silicone grease so as to bond directly to the glass despite the presence of a liquid layer (Puck *et al.*, '56). The colony isolated in this fashion can readily be trypsinized and removed without disturbance of any other cells on the plate. For maximal rigor, the cylinder may be applied a few hours after the original plating when the single cells have attached to the glass but have not yet divided. In this way, it is possible to ensure, by microscopic visualization but without micromanipulative operations, that only a single cell is trapped within the cylinder.

Cells so isolated tend to assume one of two major morphologic configurations — epithelial like and spindle shaped, or fibroblast like. These are true genetic characters, since single cells of each type uniformly breed true. The fibroblast-like cells of our experience are more sensitive to toxic agents and more demanding nutritionally than the more compactly growing, epithelial-like variety. Colonies of both types have been found in large numbers in vessels seeded directly with dispersed cells from the biopsy of a single tissue such as the lung (Puck, Cieciura, and Fisher, '57).

In early experiments, we grew single cells by plating them on top of an irradiated feeder layer. Although this was a most important development in achieving, for the first time, quantitative growth of single cells, use of the feeder technique,

for this purpose has been largely superseded by further improvement in the growth medium and conditions (Puck *et al.*, '58). The feeder technique, however, remains a most promising tool for the study of cell-cell interaction under a variety of conditions (Fisher and Puck, '56), a field of investigation that may illuminate the developmental biology of mammalian cells.

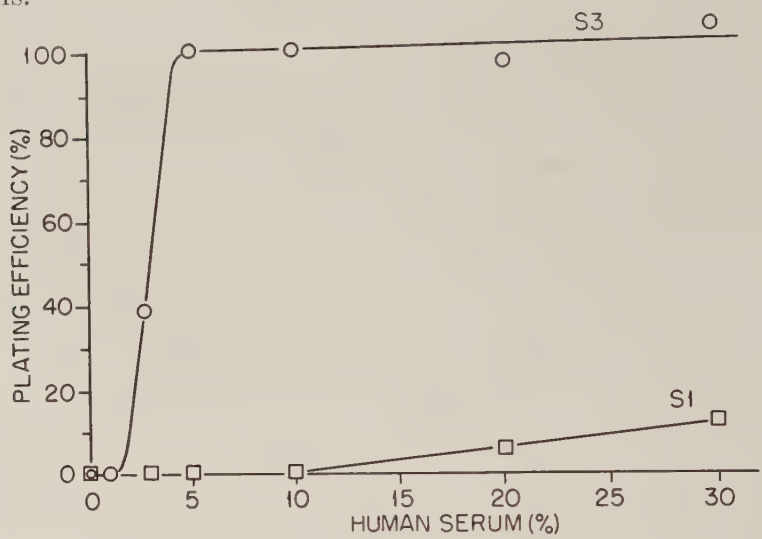

Fig. 2 Demonstration of the differential growth of cells from two different clones isolated from the same parental strain of HeLa cells.

Mutant isolation. As soon as it became evident that stable growth of single cells was reliably possible, we undertook to isolate mutants with characteristic genetic markers (Puck, '57; Puck and Fisher, '56). In addition to the obvious kinds of genetic questions that such marked cells permit, they also permitted clarification of vexing problems having to do with stability of genetic cell lines cultivated *in vitro*.

Genetically divergent forms of the HeLa cell were found in the original tissue culture stock, which is commercially supplied. These were identified in some cases by their deviant medium requirements for growth of single cells (Puck and Fisher, '56) (fig. 2). Other mutated clones were selected on

the basis of morphological variation and resistance to destruction by Newcastle disease virus (Cieciura, '58; Puck and Cieciura, '58). In addition, a variety of genetically altered forms was produced by X radiation (Puck and Marcus, '55; Puck, '57; Puck *et al.*, '57; Puck and Morkovin, unpublished results).

By means of these mutant clones, we demonstrated that clonal lines of mammalian cells with a genetic stability entirely comparable to that of microorganisms like *Escherichia coli* could be cultivated *in vitro*. Clonal strains, e.g., S3 and S1, that differ in the concentration of serum required for growth of single cells (fig. 2) and the S/NDV, a virus-resistant strain, have been maintained with unvarying behavior in rapid growth for a number of generations equivalent to 2^{500} progeny. This demonstration constituted an important step forward because, in many quarters, it had been accepted that mammalian cells grown *in vitro* are subject to so many instabilities of karyotype and metabolic behavior as to permit little definitive genetic experimentation. Examination of the chromosome complement of these cell lines has demonstrated that even these highly aneuploid cells of malignant origin can be maintained in active growth with stable karyotype over long periods, provided that the principles used routinely for stable clonal growth of bacterial and bacteriophage populations are observed. Thus the clone S/NDV after 3.1 years of continuous cultivation *in vitro* (including semiannual recloning) exhibits a population in which more than 80% of the cells show a stem-line number of 78, which is also that of the S3 and S1 clones isolated at the same time (Tjio and Puck, '58). Table 1 is a summary of all the different HeLa clones that we isolated.

Although the recurrence of the stem-line number of 78 is probably not pure coincidence, it should not be inferred that this number represents the only possible self-reproducing karyotype. In several subclones of S3 picked and developed into standard stocks by at least two consecutive single-cell isolations, other stem-line numbers have also appeared. One of

TABLE 1

"S" HeLa cell clones

	SELECTING AGENT	NUTRITIONAL SUFFICIENCY	VIRUS SENSITIVITY	CHROMOSOME STEM LINE NUMBER	COLONIAL MORPHOLOGY IN STANDARD MEDIUM
S1	—	—	+	78 [a] (Chu and Giles, '58)	Semitight
S/NDV	Virus	—	—	78 (Tjio and Puck, '58)	Semitight
S3	—	+	+	78 (Chu and Giles, '58)	Semitight
S3 Mutants:					
S3-RAI	X ray	—	+	68 (*ibid.*)	
S3-RBI	X ray	—	+	66 (Puck and H. H. Lee [b])	Giant studded
S3-REI	X ray	—	+	Mixed (Chu and Giles, '58)	Very tight
S3-R3	X ray	—	+	74 (*ibid.*)	
S3-9	—	+	+	73 (Tjio and Puck, '58)	Very tight

[a] But differs in chromosome morphology from S3.

[b] These are the results of data that are as yet preliminary but demonstrate clear difference in chromosome number from S3.

these, S3–9, produces the most uniform colonial morphologies of our experience, with cells packed extremely tightly. It consistently yields a plating efficiency close to 100%, and has a chromosome stem-line number of 73. In a chromosome analysis performed 2–3 months (equivalent to about 25 generations of growth) after its isolation, more than 90% of the cell population exhibited the stem-line number. Figure 3 shows the chromosome distribution of two of these strains.

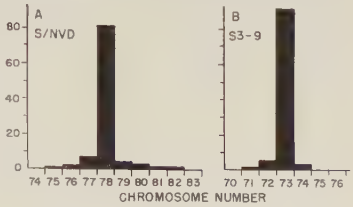

Fig. 3 Chromosome distributions in the clonal S/NDV, virus-resistant strain of HeLa cells (A) and a virus-sensitive subclone of the S3 strain (B).

Growth of single cells in a defined medium. Experiments for defining the chemical environment needed for growth of single mammalian cells *in vitro* and achieving more-precise definition of the metabolic differences between mutant forms of differing growth requirements were undertaken. Studies in other laboratories had demonstrated the need for macromolecular serum components for growth of most strains of mammalian cells and had defined many of the needed micromolecular species when massive cultures were planted, Morgan *et al.*, '50; Eagle, '55; Waymouth, '55; Evans *et al.*, '56). We found that the effectiveness of serum macromolecular fractions was highly variable and that treatment, such as prolonged dialysis, could so alter the properties of this serum fraction that it would support cell growth only when additional small molecules like cholesterol and vitamin C were added to the medium (Sato *et al.*, '57).

Careful examination of the macromolecular requirements of single S3 cells enabled us to define a medium that reliably produces 100% colony formation of single cells inoculated into a petri dish. The small molecular components of this medium have been described (Morgan *et al.*, '50; Eagle, '55; Evans *et al.*, '56; Marcus *et al.*, '56). Two macromolecular fractions were essential: serum albumin and fetuin, a glyco-

protein that constitutes a major protein of mammalian fetal serum (Fisher *et al.*, '58). Both proteins retain their activity even after extensive purification, resulting in extremely homogeneous electrophoretic and ultracentrifugal patterns (fig. 4A). Figure 4B shows results of a typical plating in which 100 S3–9 HeLa cells were inoculated into a petri dish containing purified serum albumin and fetuin and the synthetic micromolecular mixture. The availability of such a

Fig. 4 A, Electrophoretic pattern of purified fetuin, which is needed for growth of single S3 cells. Albumin used in each experiment shows a similar degree of electrophoretic homogeneity. Minor hump is delta boundary; descending pattern also contains only one moving boundary.

B, Typical plate resulting from inoculation of 100 single S3 cells into medium containing synthetic micromolecular constituents plus purified albumin and fetuin. (¾ ×)

medium now opens up many kinds of genetic-metabolic experiments with these cells. In contrast to this behavior of the HeLa S3-9 cells, many of our HeLa mutants and euploid cells from normal human tissues possess additional growth requirements, which are currently under study. Although the functions of the two needed proteins are still under study, at least part of the growth-promoting effect of fetuin may be connected with its inhibitory action on enzymic proteolysis (Fisher *et al.*, '58).

A method for systematic culture of cells from any individual, and their long-term growth in vitro without change in karyotype. Routine methods exist for transferring malignant tis-

sue of almost all kinds to growth *in vitro,* but cultivation of cells from normal adult mammalian tissues by conventional tissue culture is a relatively rare event, and even when successful, usually is only of short duration. After a few days or weeks, such cultures commonly degenerate or undergo radical transformation in chromosome constitution and other properties. Moreover, the relatively large pieces of tissue required for initiation of such cultures by conventional methods largely restrict this technique to samples of tissue removed during surgery.

We turned our attention to the development of a micro method that would reliably initiate culture *in vitro* from tissue samples of 20 mg or less, taken from human skin and equally applicable to adults or children. Thus, if growth would regularly be secured from a tissue sample small enough to permit culturing of any person, cell lines could be obtained from persons with known or suspected morphological, biochemical, immunological, or pathological genetic markers. If such cultures could be maintained in active growth for long periods *in vitro* without the chromosomal labilization that frequently occurs, systematic genetic studies could be initiated in which much of the known genetic data on man could be used.

Such a technique was developed (Puck *et al.,* '58). It is readily applicable to the skin of any person, and in a series of more than 20 such biopsies almost all have produced self-sustaining culture. Such cultures have been kept in a state of active proliferation for more than 7 months and almost 100 generations. Under the carefully regulated conditions of growth used, which involve pretesting of each batch of medium, the chromosome constitution of these euploid human cells has shown no variation (Tjio and Puck, '58). Cells taken from persons with phenylketonuria and other human genetic diseases have been established in stable culture.

Chromosomes of man and other animals. Since these techniques make it possible to sample cells from any person conveniently and reliably, it becomes a simple matter to carry out chromosome delineations on large numbers of persons. To

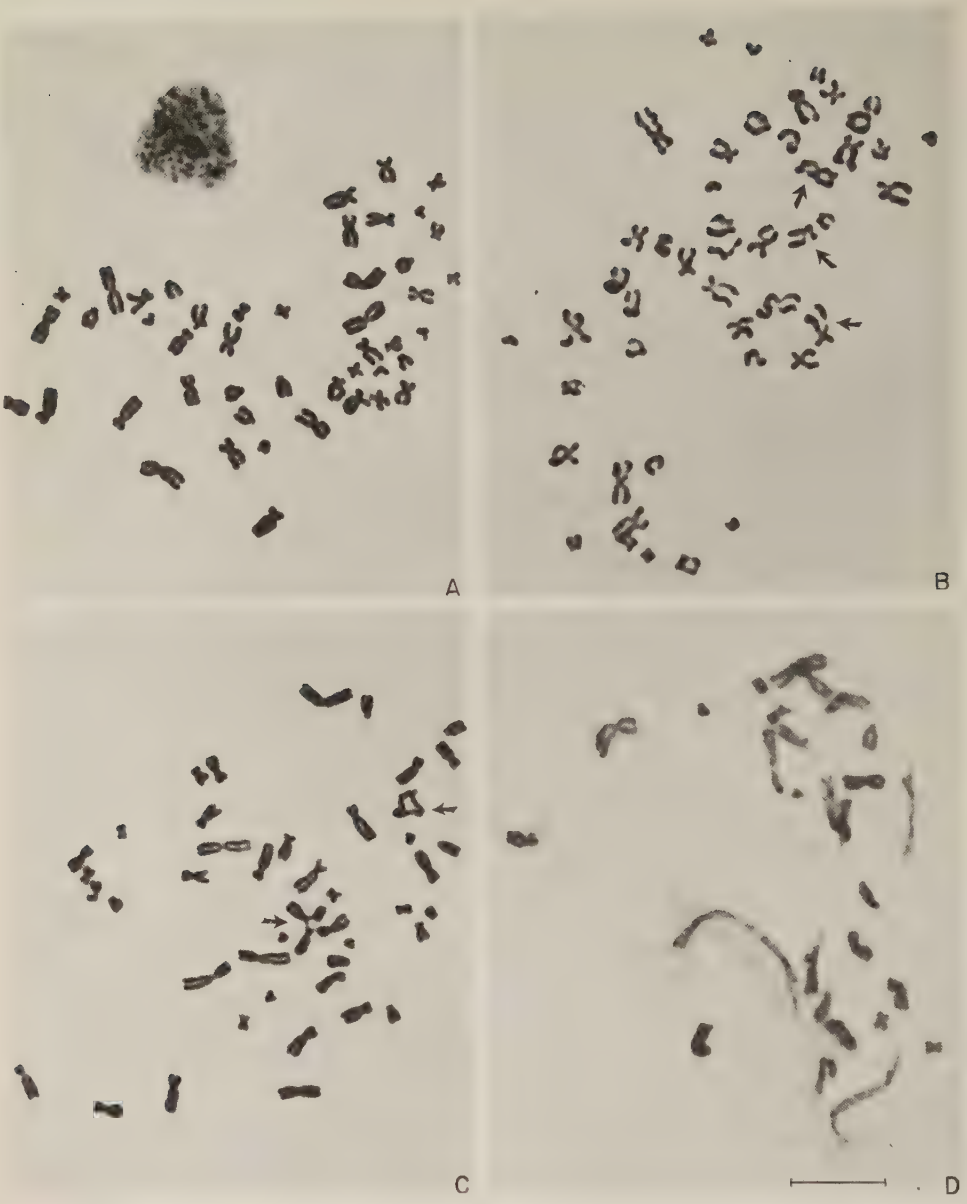

Fig. 5 Typical radiation-produced chromosomal abnormalities in euploid, fibroblast-like human cells grown *in vitro* (Puck, '58).

A, Typical set of 46 human male chromosomes from normal skin cells cultivated in tissue culture.

B, Chromosomes of cells irradiated with 50 r. At and below this dose, the aberrations consist largely of simple chromatid or isochromatid deletions, indicative of single-hit events. Typical breaks are indicated by the arrows.

C, Typical multihit aberrations produced by irradiation of euploid human cells with 75 r.

D, Sticky chromosomes produced in euploid human cells presumably by irradiation (150 r) of a cell in mitosis. Scale line is roughly 10 μ long and applies also to A, B, and C.

date, 15 human subjects have been examined (Tjio and Puck, '58). The chromosome number in all cultures of all human cells analyzed was always 46, as first described by Tjio and Levan ('56). Except for the expected morphological difference in the sex chromosomes of male and female cells and an incidence of polyploidy of about 2%, no variation in number or morphology has been observed. Figure 5A is a typical photograph. Chromosomal sex is really determined from

Fig. 6 Typical set of 22 chromosomes, cultivated *in vitro*, from normal testis of American opossum. Scale line is roughly 10 μ long.

these preparations. Experiments are in progress delineating the chromosomes of persons with known genetic abnormalities.

Chromosome delineation of cells from the Chinese hamster and the American opossum have also been carried out from stable *in vitro* culture (Tjio and Puck, '58), as shown in figure 6. The smaller number of these chromosomes gives these cells many advantages for certain types of cytogenetic studies.

Action of high-energy radiation on animal cells. These techniques have made possible for the first time quantitative determination of survival curves for the reproductive func-

tion of single animal cells exposed to toxic agents such as ionizing radiations. Typical examples are presented in figure 7 (Puck and Marcus, '55; Puck *et al.*, '57; Puck and Morkovin, unpublished results).

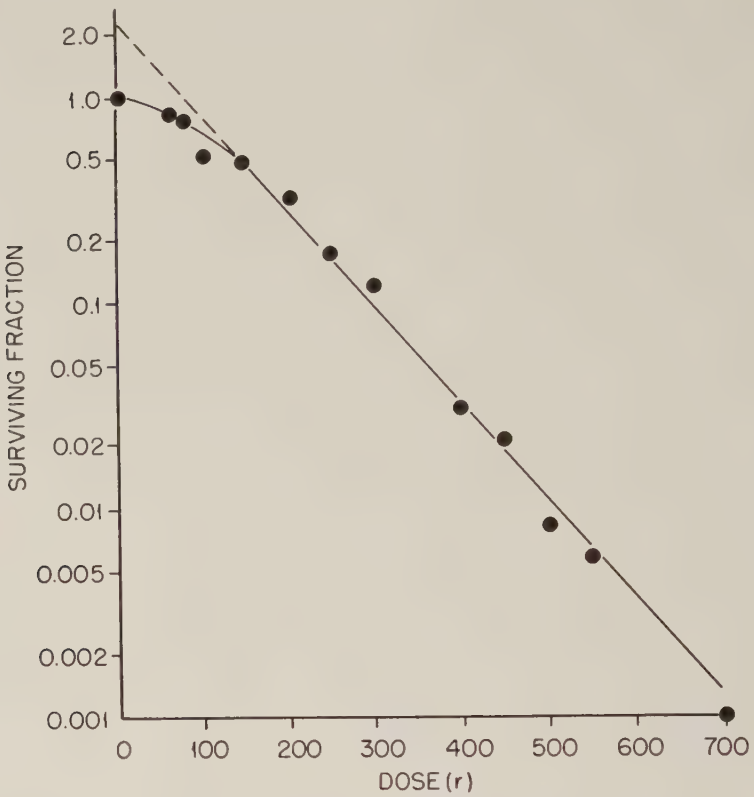

Fig. 7 Survival of colony-forming capacity of the epithelial-like S3 cells as a function of X-ray dose. The curve approximates a two-hit curve with a mean lethal dose, D_0, of 96 r (Puck and Marcus, '55). Fibroblast-like cells from normal human tissues display curves whose hit number could be either one or two, and whose D_0 value is 50–60 r.

A variety of human and animal cells displaying both normal and aneuploid karyotypes was studied and found to possess surprisingly low values for the mean lethal dose for colony formation (D_0), all the different values obtained lying within

the range of 50–160 r for mammalian cells and about 300–400 r for those of the chick. Of the human strains, aneuploid, epithelial-like cells were generally somewhat more radioresistant than euploid, fibroblast-like cells, but the duration of *in vitro* culture and the organ of origin of the cell lines examined had no discernible influence on the shape of the survival curve. Most of the animal cell lines unequivocally exhibited a hit number of approximately two; some cells yielded curves whose extrapolation to zero dose was sufficiently uncertain as to fit a hit number anywhere between one and three; and at least one animal cell we studied appears to approximate a one-hit curve (*ibid.*).

From analyses of these curves and other data, it was concluded that the primary event leading to loss of the cell's ability to multiply indefinitely is most commonly a chromosomal damage (*ibid.*; Puck, '57). By analogy with chromosomal radiation damage in other living systems, it was considered that the chromosomes of mammalian somatic cells, too, could exhibit either a generalized stickiness (if the irradiation occurred during mitosis) or chromosome breaks that could be followed either by approximately normal restitution or by the various rearrangements that have been documented in *Drosophila*, *Tradescantia*, and other forms (Muller, '54a,b). Reproductive death of irradiated mammalian cells having truly one-hit curves would result from the production of anaphase bridges from sister chromatids arising from an unreconstituted break, or from genic and chromosomal imbalance attending loss or inactivation of chromosomal material as a result of single chromosome hits. In the cells exhibiting multihit survival curves, reproductive death would occur through the intensification of these "imbalance" factors but, more particularly, through the formation of mitotic bridges and other chemical-mechanical distortions of the normal mitotic process that frequently attend the simultaneous production of two or more chromosomal breaks in the same cell. This interpretation, although demanding a relatively high yield of chromosome mutations per roentgen, explained the general

shape of the survival curves; accounted for the low magnitude of the observed lethal doses for the mammalian cells studied; correctly predicted that most of the survivors of irradiation with five to seven lethal hits would form new clones with mutant characteristics; and permitted various aspects of the radiation biology of the mammalian somatic cell to be understood in terms of the principles already established for other living forms. Further support of this interpretation was obtained from examination of the chromosomal constitution of several clonal strains of cells surviving irradiation equivalent to about six lethal hits. All four cell lines studied have exhibited chromosome constitutions markedly different from the constitution of the original unirradiated clonal stock (Puck and Marcus, '55; Puck *et al.*, '57; Chu and Giles, '58; Puck and Morkovin, unpublished results).

Cells that fail to form colonies as a result of X irradiation may reproduce once or twice. Then they usually go on growing without reproduction to form readily visible giants and achieve diameters of almost 1 mm when spread on a glass surface. Such giant formation occurs when either euploid or aneuploid cells are irradiated, but it is necessary to ensure that the growth conditions and nutrient medium provided are optimal for reliable giant formation particularly from the more sensitive fibroblast-like cells.

The interpretation proposed for the killing of cell-reproductive capacity by ionizing radiation predicts that chromosome damage should be visualizable for irradiation at doses appreciably below D_0, the mean lethal dose for reproduction. Moreover, one would expect that, whereas at radiation doses less than D_0, chromosome damage should be limited virtually to single-hit breaks, doses greater than this amount should produce multiple-hit aberrations such as dicentrics, ring chromosomes, and various translocated anomalies. This prediction was completely borne out in a study of the irradiation of a series of euploid human fibroblast-like cells (Puck, '58). The mean dose required to produce a single chromosomal hit per cell was 40–60 r in a cell whose D_0 value is 50–60 r. The

correspondence between these figures is an independent confirmation of the interpretation proposed of the mechanism for radiation destruction of the reproductive power of mammalian cells.[2] Representative pictures of radiation-induced anomalies are shown in figure 5B, C, D.

These considerations have explained a variety of phenomena, e.g., the reason why the mean lethal dose for cells such as chick fibroblasts, with much less deoxyribonucleic acid than human cells, should be much larger than that for human cells. They have also clarified various aspects of the mammalian radiation syndrome (Puck and Marcus, '55; Puck *et al.*, '57; Puck and Morkovin, unpublished results). Demonstration that the mean lethal dose for unlimited cell division represents the mean chromosome-damaging value and that various human cells possess values for this parameter in the region of 40–100 r may explain the following: (1) why some total-body radiation symptoms (such as drop in lymphocyte count) should be discernible after irradiation with only 40 r, whereas other metabolic actions not dependent on cell division (such as oxygen consumption or nerve excitation) should remain relatively unaffected by exposure to 1000 r; (2) why the mean lethal dose for an animal such as man should be about 400–500 r; (3) why rapidly multiplying tissues should first demonstrate pathological damage caused by irradiation, since chromosomal damage usually first becomes manifest when cells come into mitosis; and (4) why, after radiation exposures very near the mean lethal dose, animals can be saved by injection of viable cells of blood-forming tissue, which can continue dividing and so recolonize the region whose rapidly multiplying cells have been depleted. The correspondence in order of magnitude between the lethal dose range for cellular

[2] Bender ('57) has published a study of chromosome breaks in a human, epithelial-like cell, in which the mean dose needed to produce a chromosome break per cell was 300 r. The divergence between this figure and ours may be because his was an epithelial-like cell type but more probably is because measurements of this kind can yield only an upper limit for the value of the dose needed to produce chromosome aberrations (Puck, '58).

reproduction and that needed to suppress antibody formation (Bacq and Alexander, '55, p. 238) also strongly suggests that the depressing action of ionizing radiation on antibody production is an effect caused by inhibition of multiplication of the antibody-producing cells.

This brief account illustrates some of the different kinds of experimental questions that now can be quantitatively put to different types of mammalian cells. There can be little doubt that understanding of mammalian cell biology will grow enormously in the next several years. Although such studies will undoubtedly have many implications for practical problems involving human health and disease, the opportunities for fundamental study of cellular structure and function now possible in these systems deserve emphasis. The mammalian cell now shares with bacteria the ability to grow rapidly and stably *in vitro,* to be plated quantitatively so as to form clonal colonies, and to permit isolation of markers that can be used to study genetic processes. In addition, it is larger, its chromosomes are easily seen, and it has a much richer spectrum of metabolic activities and an enormously greater radiation sensitivity. All these properties enhance the value of the mammalian cell as a system for the most basic studies of cellular principles of organization.

OPEN DISCUSSION

Ford [3]: One thing is not clear to me. If one starts from the plating of a single cell and finds in the resultant colony some real variation of chromosome number, this must arise from mitotic irregularities during growth of the colony. On transplantation and further growth, one would expect mitotic irregularities to continue with about the same frequency and therefore an increase in the over-all range of variation of chromosome number. A constant range of variation in successive generations of transfer would then be expected only if there were regular elimination of the more extreme var-

[3] C. E. Ford, Radiobiological Research Unit, A.E.R.E., Harwell.

iants, and this would be inconsistent with 100% plating efficiency.

PUCK: One hundred per cent plating means only that the colonies produced are equal to the number of single cells plated, within the sampling uncertainty, which is about 8%. Therefore, small numbers of nonviable variants, e.g., 2 or 3%, cannot be detected, just as is true in bacterial cultures. Some of our clonal stocks possess more than 90% of the cells with a single chromosomal number. Moreover, as your own paper indicated, a small percentage of the counts on either side of the stem-line number undoubtedly represent unavoidable errors in counting.

Finally, not all the karyotypes divergent from the stem-line number are nonviable. Mammalian cells, like any other living form, are subject to discontinuous changes in their genetic constitution, most of which presumably will render the cell less able to compete with other members of the population. Hence such mutations, genic or chromosomal, may be expected to grow more slowly, perhaps, than the original form. The dynamics of such competitive population situations is sufficiently complex to permit almost any kind of constancy or change in the proportion of the different mutants present in any generation.

KLEIN [4]: When you say that you can get 60% plating efficiency of any cell type, does that include primary explants or first subcultures or do your cells have to undergo a critical period of adaptation in the culture before you get this?

PUCK: We have not been able to make any meaningful plating efficiency counts on cells taken directly from the body. In carrying out these experiments, we get apparent plating efficiencies of about 1%, but when you consider that, in removing a sample of tissue from the body, we pinch off a tiny bit of tissue with forceps, cut it into small pieces, and then expose it to various pressures and stresses, even 1% represents a relatively large number.

[4] George Klein, Karolinska Institutet.

KLEIN: How about the first subculture?

PUCK: We take the cells from the subject, place them in a petri dish with nutrient medium, and incubate them until they have grown almost to confluence. At this point, they give plating efficiencies in the range of 25–100%.

STERN [5]: My comment is concerned with the chromosome number of man. The number 46 has been mentioned exclusively by several speakers. There is much evidence for this number. Various investigators have pooled their evidence, and all agree that the chromosome number of bone marrow cells and other somatic tissue cells in Caucasoids is 46. However, I would not like to let you get the impression that this question is completely settled. Kodani, as you probably know, has reported three chromosome numbers, 46, 47, and 48, from testicular material of the Japanese.

He has a new paper in press dealing with unselected samples of testicular specimens from 15 Japanese. He finds nine with 46, one with 47, and five with 48 chromosomes. He has also investigated testicular material of eight Caucasoids from Iowa, and finds seven with 46 and one with 48. I am only reporting! We have to wait for other investigators either to confirm or to disprove the existence of more than 46 chromosomes, particularly in Oriental material.

I would like to make one more comment on this. It is at least not impossible that somatic cells will have lost the so-called supernumerary one or two chromosomes that are present, according to Kodani, in testicular cells with 47 or 48 chromosomes. Of the rather large number of white persons who have been described as having 46 chromosomes, some 17 or 20 only, if I remember right, involved testicular material. Thus, at present, 20 : 0 stands against 7 : 1 of Kodani's eight white testicular specimens.

LEDERBERG [6]: Have you begun crossing any of your mutants yet?

[5] Curt Stern, University of California, Berkeley.

[6] Joshua Lederberg, University of Wisconsin.

PUCK: We tried some experiments with a feeder layer, expecting that we might get S1 cells grown on top of the S3 feeder layer transformed into S3-like forms. This experiment produced completely negative results. We have developed a virus-carrying cell. In another experiment, we tested whether a virus grown on a nutritionally sufficient cell could transduce a deficient cell to sufficiency. So far, the results have been negative, but we believe that our tools may have been too crude. These experiments are continuing.

GILES [7]: Dr. Puck, did your successful continuous cultivation of diploid cells occur originally in a more highly purified medium than you have described to us?

PUCK: So far, the only cell that grows in a completely defined medium is the S3. Even mutants of S3 will not grow in this defined medium; we are now trying to find their specific deficiencies. All the other cells I mentioned were the fibroblast-like cells, which are more demanding. We have not yet completed analysis of the nutrient requirements of fibroblast cells. The best test of any medium is its ability to grow single cells into colonies with high efficiency. The single cell is much more susceptible to toxic factors than large cell populations. We make up large pools of medium and test them, rejecting all lots of serum with low plating efficiency. The ones that pass the test grow diploid cells stably.

WOLFE [8]: I was just wondering about attributing the two-hit survival curve to chromosomal changes; I would expect many of the wandering aberrations in your illustrations to be cell lethal, too.

PUCK: I did not mean to give the impression that a large number of breaks was characteristic of every cell that had been exposed to 25 r. We find, on the average, that 40–50 r is the X-ray dose needed to introduce one chromosomal aberration per mitosis, in euploid, fibroblast-like human cells.

[7] N. H. Giles, Yale University.

[8] H. R. Wolfe, University of Wisconsin.

Barry: In your virus-carrier cell, is there any difference in the chromosome distribution in number or in type once it is adapted to virus?

Puck: There is no difference in chromosome number. However, Dr. Chu and Dr. Giles found in examining two of our other chromosomal strains, S1 and S3, that although the distributions of chromosome numbers were the same, some differences in morphology of individual chromosomes were present. These analyses are very painstaking in a cell with 78 chromosomes and have not yet been carried out on the virus-carrier cell.

Ford: Were all the apparent chromatid breaks shown on the slides real? Some of them suggested to me the appearance that Sax identified in *Tradescantia* material many years ago and called "achromatic lesions." I have found them commonly in *Vicia* and so has Revel. They can be seen in anaphase, sometimes in one daughter chromosome and sometimes in both daughters, placed symmetrically — though a symmetrical appearance would of course derive from a chromosome as opposed to a chromatid effect. Since the presence of an achromatic lesion does not interfere with the normal anaphase movement of the whole daughter chromosome, these lesions are not true breaks. In *Vicia,* I believe Revell scores only about one-tenth of all the apparent chromatid breaks as true. So the recording of true chromatid breaks is, to say the least, rather hazardous, and it may be that some of the apparent breaks in Dr. Puck's preparations, perhaps even many of them, are not real and therefore would not contribute to the expected lethality arising from deficiency.

Puck: In referring to a "break," I mean only a complete discontinuity appearing in a fixed and stained chromosomal preparation. We rarely obtain breaks like these with unirradiated cells. This does not mean that the radiation itself is sufficient to break apart the chromosomes. All it means is that the radiation experience has changed them, so that when we subject them to the further procedure of fixing and staining they appear broken and give indication that the cells

have experienced irradiation. The fact that interaction between such breaks, such as translocations, occurs only in the dose range beyond that needed to introduce one of these "breaks" per cell, on the average, constitutes the evidence that they represent points at which chromosomes may interact abnormally with each other.

FORD: I did not make myself sufficiently clear, Dr. Puck. My reason for raising the question of the reality of many of the apparent chromatid breaks was to reply to Dr. Wolfe's point — if there had been a considerable contribution of deficiency to cell lethality, one would have expected a greater departure from your two-hit curve. But reduction of the chromatid break contribution to deficiency would mean a reduction of the linear component and so a relatively better fit to a two-hit curve.

CAVALLI-SFORZA [9]: Will you explain in a little more detail why you expect the two-hit curve.

PUCK: If a single event caused by the radiation were sufficient to destroy the cell's ability to multiply, a one-hit curve should result. Many strange things can take place in radiobiology survival curves, but they usually show up as strange kinds of curves — S-shaped curves, and so on. A curve as nice as this, with an initial shoulder that then continues to descend linearly, reasonably justifies use of a physical model, at least as a guide in further experiments. The simplest physical model that would explain this action requires the participation in the killing action of two independent radiation events. One two-hit mechanism that will not fit the data would attribute cell death to inactivation of the same gene on two sister chromosomes of a diploid cell. Such a mechanism would require a much higher D_0 value. However, one can get good agreement with this curve by the hypothesis that any two chromosomes simultaneously hit have a high probability of destroying the reproduction of that cell.

HAUSCHKA [10]: Dr. Puck, a 50% lethal radiation dose of only 94 r applies (according to one of your recent publications)

[9] L. L. Cavalli-Sforza, University of Pavia.

[10] T. S. Hauschka, Roswell Park Memorial Institute.

not only to HeLa cells, which have 78 chromosomes, but also to three normal human cell strains that Dr. Tjio (in your laboratory) finds to be diploid. How could four cell strains (one subtetraploid and three near-diploid) respond identically to produce a straight-line response when the percentage cell survival is plotted against increasing doses of radiation?

PUCK: They are approximately but not exactly the same. I indicated that such cells had mean lethal doses contained within the range of about 50–150 r. This is a very small range compared to the values of 20,000 and 200,000 r that used to be claimed as the mean lethal dose of mammalian cells *in vitro*. On the other hand, this is a 3:1 difference and is indeed significant. The larger the number of chromosomes and the larger their volume, the greater the probability of this kind of essentially nonspecific chromosome lethality, and this is exactly what is found. Other factors undoubtedly have an influence, but it is noteworthy that cells of the Chinese hamster, which have only 22 chromosomes, and of the chick fibroblast, which has much less DNA than human cells, are the most resistant that we have found.

AUERBACH[11]: If I may join the discussion of the "hit curve"—this question is quite familiar to *Drosophila* geneticists dealing with dominant lethals. The percentage of dominant lethals plotted against dose gives a curve that starts linearly, then becomes exponential; and finally, of course, one gets saturation. During the linear part, death is caused by single chromosome breaks, probably owing to sister chromatid fusion and chromosome loss. Later on, there are two-hit events presumably in the main inviable translocation. I agree that the chromosome number is decisive, but I would say not in determining over-all resistance but rather in determining which part of the dominant lethal curve is important. With more chromosomes per cell, the production of translocations is facilitated. It seems that in the mouse, with 20 chromosome pairs, a high proportion of dominant lethals

[11] Charlotte Auerbach, Oak Ridge National Laboratory; on leave from University of Edinburgh.

induced by irradiation are caused by two-hit events. So I would expect that with human cells, especially with the polyploid HeLa cells, the majority would be attributable to two-hit events. I would be interested to know whether the curve has a stronger linear component in the hamster with its small chromosome number.

PUCK: The curve with the Chinese hamster is also two-hit.

HARDY: Have you any idea about the mechanism by which the factor allows the cells to attach themselves to the grafts and in what way the attachment of the cell is related to the growth of the cell? I mean, what is primary and what is secondary.

PUCK: Fetuin is necessary for the attachment of cells on glass and also for their growth, but growth requires fetuin even when the cells are in suspension. If these cells are put in a paraffin-lined vessel where they cannot attach under any circumstances, they will multiply in the presence of fetuin but not in its absence. So fetuin changes the surface of the cell in such a way that it can do two things. It can stick to a glass surface if it is available; but, regardless of whether a glass surface is available, it can multiply.

YERGANIAN [12]: I was surprised to hear that individual cells of the Chinese hamster are quite radioresistant. Some time ago (Yerganian, '55), an $LD_{50}/30$ determination was undertaken with surplus adult animals. Two X-ray sources were used. The first had the following radiation factors: 150 kv, 7 ma, 100 r/minute in 25 cm of air, and an inherent filtration equivalent to 0.5 mm of Al. The $LD_{50}/30$ was 1200 r, as compared to 880 r and 760 r for Syrian hamsters and rats, respectively, determined by colleagues using identical factors. A deep-therapy unit operated at 250 kv, 15 ma, 55 r/minute in 50 cm of air, and with added filtration of 2 mm of Cu and 1 mm of Al, resulted in a $LD_{50}/30$ of 825 r. Mice and guinea pigs exhibited the expected dose-mortality responses. A tentative conclusion, based on the above whole-body X-irradiation data and that presented by Dr. Puck for single cells, suggests

[12] George Yerganian, Children's Cancer Research Foundation, Boston.

the presence of intracellular elements other than nuclear targets and humoral factors that offer protection against X-ray damage or encourage rapid recovery.

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General Discussion

MOLECULAR BASIS OF THE CAUSE AND EXPRESSION OF SOMATIC CELL VARIATION

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ONE FIGURE

TATUM: In a discussion of the molecular bases of variation, we are concerned primarily with three different problems that have to do with information at one level or another: (1) the molecular basis of storage or coding, (2) information transfer from cell to cell, and (3) translation of information into phenotypic expression.

In all three areas we are concerned with the molecular relation of ribo- and deoxyribonucleic acids (RNA, DNA), protein, and other cellular constituents in a cyclic, dynamic, and very complex system. We hope to bring out some exam-

ples of molecular specificity in coding, translation, and expression of information. We also hope that some of the molecular bases of enzymic activity, enzyme formation, and so on, to be discussed here will give models that we can try to apply to the more difficult and subtle problems of variation and differentiation.

A gene essentially determines the information that is available. Dr. Brink and others in this symposium have discussed the possible role of gene mutation in this primary sense in cell variation. The expression of this information is affected by various factors, however, including environment. In addition, secondary gene mutation itself can lead to varying expressions of primary genes. The activity of a particular gene can also be specifically changed by other modifying and suppressor genes, so that it becomes more or less susceptible to the environment. This action may come at any place in the long sequence between the primary gene and its final phenotypic expression.

It should be pointed out also, that we may tend to oversimplify the picture because we consider a cell as a unit with nutrients coming in and waste products going out. A cell is not an isolated unit any more than a gene is. The expression of its genic information is influenced by the adjacent cells, as Dr. Puck has described, in a nutritional sense. Hence cells in a uniform, homogeneous state in a culture may express either more or less of their genetic potentiality than in their natural environment, so that we cannot necessarily deduce their activities during development from the activities of those in homogeneous culture.

For convenience, we have arranged a sequence of general topics for discussion; first, the gene and its behavior; second, chromosomal and nuclear variation and translation of this information into enzyme specificity; and third, the possible role of enzyme differentiation and enzyme induction in variation and development.

Dr. Mazia will discuss the role of the nucleus in cell variation.

MAZIA: Our mission is to discuss the molecular basis of the *expression* and *cause* of somatic cell variation and to identify those expressions of variation that are most relevant to their causes, to genetic and epigenetic operations.

Curt Stern opened our conference with a summary of evidence that cell differentiation might be associated with modifications of the nucleus, which, in view of its administrative function, should be given a high priority among the possible causes of cell variation. But what may be the molecular basis of nuclear variation?

Representation of the primary genome by DNA is supported by its quantitative constancy and metabolic stability. But there is now evidence that at least part of the DNA may be variable in amount (Rudkin and Corlette, '57) and metabolic activity (Ficq and Pavan, '57). It has been proposed that the variable fraction may be associated with gene action, for it is associated with particular chromosomal sites. Here, then, is one potential chemical source of cell variation.

Another nuclear variable that might be relevant is the distribution of enzymes. Mirsky and his collaborators (Allfrey *et al.,* '55) described characteristic patterns of intranuclear enzymes that are correlated with the tissue types from which the nuclei were isolated and stressed the possible correlations of this finding with differentiation.

Earlier in these discussions, Dr. Gelfant mentioned the nonhistone proteins of nuclei. These are not constant in relation to DNA but vary from one kind of cell to another. They also vary with the functional state of a cell, and so may represent a result rather than a cause of the stable differences between cells. Finally, I should mention variations in nuclear RNA that may serve as a chemical mirror of differences in the decisions being made by nuclei.

This, then, is a descriptive basis for extending the study of nuclear differences, as presented by Dr. Stern, to the chemical level. Our knowledge is fragmentary, and we must remind

ourselves constantly that difference is not automatically equated to differentiation.

In attempting to visualize the method — I hesitate to use the word mechanism — of nuclear differentiation, we are required to find a basis for asymmetry in mitotic division. The classic paradox, of course, stems from our conception of mitosis as a device that guarantees nuclear identity in the daughter cells. In many situations we do observe that differentiation is established by one cell division, as in meristematic tissues in plants, or in stem lines in animals. Can we find a basis for asymmetry in mitosis, preferably one that will not violate the precept of the equipartition of the primary genome?

Recent cytochemical work opens up some possibilities. An important addition to our picture of mitosis is discovery that the chromosomes distribute RNA. A number of workers, beginning with Kaufmann *et al.* ('48) have shown that the chromosome acquires RNA during prophase and apparently dumps it into the cytoplasm at the end of anaphase. Chromosomes may similarly direct the distribution of still other substances between the daughter cells.

But how can we imagine the origin of an asymmetry in the distribution of chromosomal substances? I should like to propose a principle whose reasonableness will depend on the method of duplication of the chromosomes. When a chromatid duplicates, is its substance divided equally between its daughters? Or does the parent chromatid retain all of the old substance and make a new daughter? Dr. Plaut and I ('56) have obtained results that favor the second possibility, and it is supported by further work that he has done. However, the results of Taylor *et al.* ('57) can be interpreted as meaning that the parental DNA is distributed equally between daughter chromosomes. Without considering the question to be settled, let us examine the consequence of the hypothesis that chromosome duplication is a process wherein a stable old chromosome produces a new daughter. [Ed. note: Later discussion of the pertinent and important work of M. Meselson

and F. W. Stahl, following N^{15}-labeled DNA through cell division in *Escherichia coli,* is omitted since the work was then unpublished.] It is simply this: of a pair of sister chromosomes separating at mitosis, *only one was synthesized during the preceding interphase* and, therefore, only one was subjected to variables of the internal and external environment that might affect its synthesis. If conditions of synthesis determine the composition of the chromosome, including components other than DNA, the daughter cells will not necessarily receive identical chromosomes. This would, it seems to me, provide a workable design for achieving a chemical differentiation between daughter nuclei in a single division.

We know that daughter nuclei may differ chemically even when they remain in the same cytoplasm. The two nuclei of the pollen grain differ greatly in protein content even though their DNA contents are identical (Bryan, '51). Can we detect a chemical asymmetry between sister chromosome sets even during mitosis? Cytochemical tools are becoming available for such a search. The very experimental designs that have shown DNA to be equally distributed, within present limits of error, could be refined for possible detection of small inequalities of DNA or inequalities of the protein and RNA constituents of the chromosomes in divisions in which the daughter nuclei will have different fates.

In conclusion, I should like to repeat the caution that *difference* is not to be equated to *differentiation.* Even if the difference is detected in the inner sanctum of the nucleus, we still have to prove that it possesses authority.

STREHLER [1]: Dr. Mazia's suggestion that a segregation of cytoplasm may take place during cleavage may be well taken for certain types of regulative eggs, but in highly mosaic eggs (e. g., Diptera) a localization of presumptive organ areas and presumably chemical differences exist even prior to cleavage.

It is apparent that these differences become more pronounced in the differentiation of highly specialized cell types. As a model for senescence we are interested in the hypothesis

[1] Bernard L. Strehler, National Institutes of Health, Baltimore City Hospitals.

that an unrestricted extension of normal chemical specialization (differentiation) of metazoan cells will lead to their death. For example, assume that in muscle there is some optimum functional concentration of contractile protein as compared to supporting elements (both metabolic and structural). In the undifferentiated state, this percentage might be 10% ; in the young muscle cell, 50% ; and in the young adult, 90%. A continuation of an automatic undamped differentiation process to be described would produce nonviable cells (e. g., 95% contractile proteins).

Assume two cells arising by cleavage from a single egg cell. Cell A may contain two units of component (Aa) and one of component (Bb). Cell B, by contrast, contains two units of (Bb) and one of (Aa). (A and B are protein; a and b are the corresponding nucleic acids.) Assume that the rate of synthesis of Aa and Bb is proportional (first order) to the concentration of Aa and Bb. Now, as long as A and a (B and b) remain tightly associated, the rate of synthesis of Aa and Bb will be such that the relative concentrations will not change (the growth phase).

However, consider the result if the nucleoproteins in question begin to dissociate (owing to pH change?) when growth ceases as a consequence of exhaustion of stored food (e.g., glycogen). If synthetic rate remains proportional to the specific (associated) nucleoprotein concentration, then those systems enjoying an initial concentration advantage will swamp out the lesser components because the concentration of specific active synthetic units (combined form) will be a squared function of the concentration of their component parts. In other words, differentiation would ensue if, as a result of a dissociation of nucleoproteins, the synthetic processes shift from a first- to a second-order autocatalytic reaction.

The equation (Strehler, unpublished) relating synthetic rate (assuming other factors are not limiting) to the total concentration

of a specific protein $[A_t]$ and the nucleoprotein dissociation constant K is:

$$\text{Rate of synthesis of Aa} = d\,[A_t]/dt = k_s\,[Aa]$$
$$= k_s \left\{ [A_t] + K/2 - K\sqrt{([A_t]/K) + 1/4} \right\}.$$

MAZIA: The analogy between the general situation in which differentiation is determined at one division and that in a mosaic egg would work nicely. But, if we think of the many situations in which the asymmetric divisions may take place with high frequency, where a stem-like population is throwing off great numbers of cells that will have a different history from the parental type, we would have to imagine that the process of cytoplasmic segregation was taking place in each generation. It seems a little clumsy, but it is a known method for obtaining nonidentical daughter cells at one division, even if we know nothing about how the regional differences are established and how they determine the plane of division.

GELFANT [2]: I do not mean to depreciate the role of RNA as Dr. Mazia has pointed it out, but the evidence is mainly cytochemical. I wonder, therefore, if the role of the nonhistone protein really might not be the most important factor.

MAZIA: The point made by Dr. Gelfant is appealing, especially since we do know of cases, such as the pollen grain, in which sister nuclei differ greatly in their protein content. But I think that the chromosomal RNA cycle should not be overlooked. Perhaps it is related only to the mechanism of mitosis, the coiling of chromosomes, for instance. But it is a striking fact that the chromosomes pick up RNA at the time of division and carry it to the daughter cells.

TATUM: I think we had best move on then to an area in which perhaps we have a little more knowledge on a molecular basis; that is, the translation of genetic information into enzyme synthesis. I would like Dr. Spiegelman to introduce this subject.

SPIEGELMAN: As I understand our function, it is to attempt to provide a basis of departure for a discussion of somatic

[2] Seymour Gelfant, Syracuse University.

variation in molecular terms. I should like to make a plea for an optimistic view of morphogenesis based on a belief in simplicity.

The mechanisms underlying differentiation are in large part, I think, understandable in terms of chemical components already known and identified. We are living in the era of the Watson-Crick model in which experiments performed, or on the verge of execution, will lead to complete elucidation of the central problems of biology, the mechanism of duplication. This same era has seen the successful isolation in cell-free state of enzyme systems that can fabricate such complicated molecules as DNA, RNA, and protein. It is no longer relevant to phrase questions of cell physiology in terms of other than defined chemical entities. It seems to me that the same is true for morphogenetic events.

I should like now to describe a simple enzymically defined system that leads to heritable differences of enzyme-forming ability in cells of identical genotypes. This stems from Novick's analysis of a peculiar sort of induction of β-galactosidase in *E. coli.* This system resembles in many ways one that we investigated in yeast about 10 years ago under the name of long-term adaptation.

Under conditions of saturating inducer levels, a linear relation is found between the appearance of new β-galactosidase and the synthesis of new protein. Immediately upon addition of the inducer, enzyme synthesis proceeds at its maximal relative rate. This means that a constant proportion of the new protoplasm formed is the enzyme in question and also suggests that all the cells participate uniformly in the synthetic process.

This simple picture is not observed at all inducer concentrations. If the inducer level is dropped below saturating values, there is a lag period prior to enzyme formation, followed by a period of accelerating synthesis that ultimately reaches the rate observed at higher levels of inducer. Novick found that at low inducer levels the accelerating phase of

enzyme formation was ascribable to a heterogeneity in the population. At any given time, a certain proportion of cells were capable of carrying out the inductive process and handing on this ability to their descendants. The remainder of the cells were unable to form enzyme and neither were their descendants. These two cell types can continue to exist and multiply for many generations living side by side in the same medium and retain this heritable difference. In long-term adaptation in yeast, we were easily able to show that the difference in enzyme-forming capacity was indeed heritable by using indicator plates on which clones growing up side by side were easily distinguishable.

The mechanism of this heritable difference in *E. coli* turns out to be simple and is probably based on a single enzymic component. If the external inducer is to get inside, a transport enzyme, or permease, has to be formed. Furthermore, the inducer once inside the cell acts as an inducer of the permease as well as of β-galactosidase. At low levels of inducer every cell has a low, fixed probability of spontaneously forming a molecule of permease. Once a molecule of permease is made, however, more permease molecules will soon be synthesized since its existence leads to the ready transport and concentration of inducer molecules. Thus enzyme-forming ability is autocatalytically built up and transmitted to descendants in a cytoplasmic manner.

By this comparatively simple device a heterogeneous population of cells that differ in their capacity to form the β-galactosidase is obtained. Kinetically, this process is exactly equivalent to mutation, and can be measured by the same devices.

I am not proposing that such relatively simple mechanisms are predominant in cell differentiation, but they illustrate that enormously complicated schemes are not necessary to mirror some schemes observed in embryogenesis.

I should like to make one other point about mechanisms of this kind. In the permease system and apparently in the

long-term adaptation case, the kinetics observed were consistent with the assumption that the possession of one particle was sufficient for the cell to retain heritable ability to form enzyme. This raises a problem relevant to Professor Ephrussi's question, how do we differentiate between steady-state and particulate systems? We normally think of steady-state devices in terms of enzymically controlled reactions. It is evident from the preceding discussion that, as soon as the number of relevant enzyme molecules falls to levels of the order one, the system will assume particulate properties. Under the circumstances, it seems more pertinent to phrase these questions differently. When we inquire whether a heritable system is steady state or particulate, we are primarily concerned with the relation of the heritable characteristic of the system and the structure of the elements involved. If an element uses its structural units as ordering devices for replication of another element like itself and thereby maintains the character of the system, we would call it particulate. If, on the other hand, an element maintains the relevant property by using some component part (e.g., active center of an enzyme) to maintain the concentration of some compound, we would call it steady state. In modern parlance, what we are asking is whether or not the key element contains nucleic acid.

Ephrussi[3]: I mentioned this mechanism because I think it is a very important model — so important that we should not over-emphasize it. We have here a very interesting case of apparently intrinsically identical cell lines that may be continuously maintained in different states. However, it should be mentioned that this remains so only so long as the cells are in the presence of a very low level of the inducer, thiomethyl-β,D-galactoside (TMG), and that, as soon as TMG is omitted, the phenomenon is gone and you have a simple dilution of the enzyme. This is a real difference between this system and that which we think we have in differentiation,

[3] Boris Ephrussi, University of Paris.

where we put two cell lines into a completely heterologous medium. We have no evidence for the presence in this medium of a specific inducing substance, and yet the phenomenon persists.

SPIEGELMAN: I agree that mechanisms such as I have discussed probably do not play a decidedly dominant role in embryogenesis. However, differences in β-galactosidase-forming ability within a population have been maintained for over 80 cell generations by using what are called maintenance levels of glucose. Eighty cell generations is virtually an infinite time as far as morphogenetic phenomena are concerned, and to this extent it seems to me the parallelism is good enough.

TATUM: In elaboration of this model, it should be pointed out that such an initial heritable differential in metabolism of a single compound, could, by a process of internal sequential induction, lead to acquisition of many other metabolic differences.

NOVELLI: I would like to discuss the suppression (or repression) of enzyme synthesis as another model system that can function to regulate the metabolic activity of a cell. Enzyme suppression is the peculiar behavior of the end product in a long pathway of biosynthesis that feeds back to regulate the synthesis of enzymes involved in its own formation. This phenomenon was first observed by H. Vogel in *E. coli,* in which arginine suppresses formation of acetylornithinase, which is necessary for arginine synthesis. Subsequently, additional examples of this phenomenon were studied by W. K. Maas and L. Gorini, H. E. Umbarger, A. B. Pardee, and B. Magasanik.

The particular example I want to discuss is the case of ornithine transcarbamylase (OTC) in *E. coli* described by Maas and Gorini. The pathway used by *E. coli* for synthesis of arginine starts with glutamic acid → N-acetylglutamic acid → N-acetylglutamyl semialdehyde → N-acetylornithine → ornithine → citrulline → arginine. OTC catalyzes the conversion of ornithine to citrulline. Maas and Gor-

ini demonstrated that the level of OTC in the cell is regulated by arginine. Thus, if cells are cultivated in the presence of arginine, little or none of this enzyme is formed. (Several other enzymes in this biosynthetic chain are similarly suppressed by arginine.) This suppression acts to block biosynthesis of arginine, and the cells grow at the expense of the arginine supplied in the medium. If such cells are removed from the arginine medium and are resuspended in growth medium in the absence of arginine, there is a rapid burst of OTC synthesis reaching a maximum just before the first generation. The consequence of this new enzyme synthesis is that the biosynthetic pathway to the formation of arginine is opened up and the cells start making their own arginine. As soon as the internal concentration of arginine builds up, further enzyme synthesis ceases and the enzyme concentration is maintained at the steady-state level. Thus the internal level of arginine regulates the level of enzyme that the cell needs. Any change in internal arginine concentration that may be brought about by demands made on the cell can be easily adjusted by this feed-back mechanism that controls the enzyme level.

The combination of substrate-inducible enzymes (discussed by Spiegelman) and enzyme suppression through the negative feed-back effect provide model systems for discussion of differentiation at the molecular level. It is known that the enzymic composition of cells changes as they undergo differentiation, but it is not known whether the change is cause or result of differentiation. However, these two model systems provide a mechanism whereby such changes can be brought about. Careful examination of these phenomena in cells undergoing differentiation may lead to a better understanding of the initiation mechanism of the process.

Luria [4]: I would like to point out a limitation to the analogy between microorganisms and somatic cells. One of the main goals of microorganisms for success seems to be that

[4] S. E. Luria, University of Illinois.

of regulating the best possible utilization of substrates and environmental situations *for growth,* whereas, in differentiation of cells in a metazoan, the main problem may be to make the best of a certain environment for a certain specific function. possibly the essential mechanisms here are those that *suppress* or stop the phenomena that make for the best possible growth and unleash instead the synthetic possibilities for some rather unique product that the cell has to make. A specialized cell that has lost some of the highly refined mechanisms of regulation for maximum growth would be a wasteful cell by the standards by which we judge "successful" microorganisms.

TATUM: I think we have an opportunity now perhaps to extend some of these concepts and to look a little more specifically at the relation between enzyme synthesis and activity in connection with development in animals. I would like to ask Dr. Knox to present his views on this.

KNOX: Since I am to discuss animals, let me give a frame of reference.

During embryological development as the cells are morphologically differentiating, say, in the liver, there is an orderly appearance of different specific enzymes whose levels fluctuate in a prescribed manner. There are many of these enzymes, and by the end of gestation the metabolic equipment in the liver is somewhat like that present in the adult animal. In general, there is a 1:1:1 correlation of morphological differentiation, appearance of specific enzyme activity, and the total physiological activity of this organ.

The literature contains some quite striking examples of this. For example, xanthine oxidase in the liver of the chick embryo is absent before the chick has put a hole in the eggshell and is present in the full adult concentration afterward. Kretchmer and McNamara ('56) report absence of tyrosine transaminase in the human baby at birth and the newborn rat and its presence 4 hours after birth in nearly adult concentration.

My frame of reference is simply that these changes in specific enzyme activity in an organism constitute biochemical

differentiation. How this fits into morphological differentiation is another problem, which would take us too far afield. But we can ask what kinds of mechanisms could be responsible for these quite dramatic changes in enzyme concentration.

Here I can talk about the adult animal, which we have actually studied. We can say that the same types of mechanisms that operate in microorganisms to change enzyme levels also operate in animal cells. There can be induction of an enzyme by its substrate. Tryptophan peroxidase, an enzyme limited to the liver, is induced 5 hours after ingestion of its substrate to a maximum of some ten times the basal concentration. The excess enzyme falls to the normal level when the excess tryptophan is metabolized. There are also negative suppression effects very much like those referred to in the arginine series. Cholesterol administered to an animal will result in the suppression of cholesterol synthesis, not only in the intact animal but in the mitochondrial system isolated from the cholesterol-fed animals. This has been demonstrated by the experiments of N. L. R. Bucher, among others.

There is also a sort of coarse control over and above this fine control of induction of a single enzyme by its substrate. Tryptophan peroxidase and several physiologically related enzymes can all be induced by a hormone. Hydrocortisone from the adrenal glands will induce tryptophan peroxidase, tyrosine transaminase, and a series of enzymes involved in amino acid metabolism. I have no idea how hormones do this in terms of chemical mechanism, but I have very little doubt that the physiological effect of hormones is manifested by this changing of the enzyme concentrations in the cells. According to our current picture, a substrate will act to induce its specific enzyme and a hormone will act to induce a series of physiologically related enzymes. This is a very simple two-way mechanism, but I have neglected the factors that determine the responsiveness of the organism.

We have grouped other determining factors under the label of "metabolic state." Perhaps the easiest way to define this

is simply to say that the organism in two different physiological states will not respond to the same inducer in the same way. This is quite obvious, since the metabolic machinery is going to be different in different states. It is not just that mice are different from rats, that males are different from females, or that liver is different from kidney. The liver of an adrenalectomized rat is different from that of an intact rat and may well respond to the same stimulus in quite different ways. For example, adrenalectomy fails to cause any change in the glucose 6-phosphatase level in the liver of the intact rat. The diabetic rat liver has an increased glucose 6-phosphatase concentration, and in that animal adrenalectomy reduces the level to normal.

Although we know that in the adult stage these several factors (substrate concentrations and hormone concentrations interacting with the metabolic state of the cells) can cause very large increases and decreases in a short length of time in the amount of specific enzymes, does this help us at all in understanding the changes that take place during differentiation? I think so, but we can speak with knowledge only about the changes that occur near the end of gestation. Tyrosine transaminase, which is absent in the liver of the newborn child and the newborn rat and present in nearly full concentration 5 hours after birth, can be induced by hydrocortisone from the adult level to a level about ten times as great. It is at least possible that the neonatal induction of tyrosine transaminase is also brought about by hydrocortisone, since we know this hormone is released from the fetal adrenal gland at birth, and since we would expect it to have its maximum effect on this enzyme 5 hours later.

Tatum: Would you care to speculate on this use of the term "induction" for hormone stimulation or on the mechanism by which hormones act?

Knox: We have studied the effects of hydrocortisone and tryptophan on induction of liver tryptophan peroxidase quite extensively, and I think we can rule out any changed penetra-

tion. These experiments consisted in measuring the free concentration of tryptophan in the liver cell during induction by either substrate or hormone. We did this to test the hypothesis of changes in tissue permeability but, more importantly, to examine further the rather striking discovery that hormones are inducers of enzymes. Hydrocortisone causes a certain amount of protein breakdown in the body and could induce the enzymes secondarily by increasing the level of the free tryptophan. Measurements of the free tryptophan concentration showed that induction by hydrocortisone occurred without any change in free tryptophan concentration in the liver cell or any increase in metabolites of tryptophan collected from pyridoxine-deficient animals.

So the problem of mechanism is not very different from that in microorganism, where compounds that are not substrates also can act as inducers. Microorganisms have been offered compounds quite closely related to the substrates, whereas we are going farther and offering compounds like steroids.

Spiegelman: A few words may perhaps be said about the present status of the mechanism of induction. One of the most dramatic features about enzyme induction is that we can turn on and off the synthesis of specific proteins by introducing or removing from the environment relevant chemical compounds. The phenomenon, in itself, has been exploited and used quite intelligently for the study of protein synthesis. But the sad fact remains that we really know as little about the chemical mechanism of induction and the metabolism involved as we did when a renewed investigation of enzymic adaptation was begun something like 15 years ago.

Gelfant: I would like to know if Dr. Knox considers that the increase or decrease of an enzyme in a tissue after administration of a hormone is proof of the action of that hormone on that enzyme.

Knox: I would say that its action is not on the enzyme itself. All the effects mentioned occur in the absence of any effects of the inducing substances on the cell-free enzyme. We

look upon the enzyme changes, I think with quite good evidence, as increases in the actual amount of specific protein. What we are measuring is enzyme accumulation, either by increasing the rate of synthesis or decreasing the rate of breakdown.

KRETCHMAR[5]: The work of Christensen is pertinent to the discussion of the effect of hydrocortisone on protein metabolism in the liver. Aminoisobutyric acid was found in increased concentration in liver cells as early as 2 hours after a dose of hydrocortisone. We have used the Hamilton-Van Slyke ninhydrin procedure to study the effect of hydrocortisone on free amino acid nitrogen in liver, plasma, and muscle. The earliest effect was a depression of plasma levels. At a later time there was an increase in the amino nitrogen level of liver cell water. No changes occurred in muscle over the period of 24 hours. This evidence suggests that hydrocortisone increases the concentrative transfer of amino acids into the liver cells.

HEIDELBERGER: I would like to talk about a few very simple organic chemical relations of immunologically specific polysaccharides and, in particular, of the blood group substances that figured in some of the discussions in this symposium.

As you remember from work of E. A. Kabat and others on the blood group A specific substance, for example, this has a backbone structure of galactose, N-acetylglucosamine, and N-acetylgalactosamine, with a number of side chains of furanofucose. Kabat and his coworkers showed that, if the furanofucose side chains of the blood group A substance were split off by mild acid hydrolysis, the cross reactivity in type-14 antipneumococcus serum went up enormously.

Now our knowledge of the fine structure of the specific polysaccharide of type-14 pneumococcus, which is the determinant of type-14 specificity, is *very* recent and came partly from Birmingham, England.

[5] Arthur L. Kretchmar, Oak Ridge Institute of Nuclear Studies.

If the main chain of the blood group A substance is composed of galactose, N-acetylglucosamine, and N-acetylgalactosamine, we can readily understand why galactose and N-acetylglucosamine stretched out in a chain would give cross reactions with the blood group A substance. When the main chain of the blood group substance, whose exact nature we do not know yet, is laid bare by removal of the side chains of furanofucose, we have liberated multiple reactive groupings that can react more readily with antibodies to another substance composed in large part of galactose, N-acetylglucosamine, and glucose and in which portions, at least, of the first two sugars, must occur in the same linkages.

There are many other polysaccharides containing galactose that also give cross reactions in type-14 antipneumococcus serum. We have found that every polysaccharide with multiple nonreducing end groups of galactose precipitated a fair proportion of the antibodies in type-14 antipneumococcus serum. We could deduce from this that, when the fine structure of the type-14 pneumococcus polysaccharide was elucidated, it would be found that part of the galactose, at least, would be in the form of such end groups. About a year ago, Dr. S. A. Barker, who is working on this in Birmingham, England, actually isolated tetramethylgalactose from the methylated type-14 substance, proving that there were nonreducing end groups just as indicated by the serological cross reactions. Now he has gone much further through partial hydrolysis of the type-14 polysaccharide. He has isolated two disaccharides, one of which appears to be glucose $\beta,1 \rightarrow 4$,N-acetylglucosamine, and, since the glucose is all accounted for in the methylated polysaccharide as nonreducing end groups, it is clear that this determines the two end sugars on the main chains of the type-14 pneumococcus polysaccharide. The polysaccharide is very highly branched, and there are more side chains ending in galactose stretched along the main chain than there are glucose end groups.

Dr. Barker also isolated another disaccharide that appears to be galactose $\beta,1 \rightarrow 3$,N-acetylglucosamine. That has not

been definitely decided yet, but the main features of the structure of the type-14 polysaccharide work out in this way. Although this is not an absolute formula, it is one of a limited number of alternatives that are highly probable.

This, in turn, goes back to the structure of the blood group A substance, because it is quite clear that, since there is no N-acetylgalactosamine in the type-14 substance, the galactose-N-acetylglucosamine portions of the molecule must be very closely related. It is quite probable that, when the fine structure of the group A substances is worked out, it will be found to have this other disaccharide linkage, galactose $\beta,1 \rightarrow 3$,N-acetylglucosamine, repeated a number of times in its principal chain or chains. In this way I think we are getting down a little closer to the actual molecular structure of some of the end products of genic action.

Hotchkiss: In this symposium we are in part concerned with how an aggregate of many genes gives rise to an aggregate of many enzymes, many macromolecules containing specific structural proteins and carbohydrates as well as specific enzymes. In talking about the things we may hope that chemistry can do for the study of growth and differentiation, I shall proceed to take an over-all look at the aggregate of processes in a living cell. We might think of the whole process of the life of the cell as matter on a time axis, going through metabolism.

Usually we consider some special category of matter, such as a particular substance, and follow its mass at a series of points along the time axis. In this way we deal with the formation, the biosynthesis or the oxidation and so on, of chemical constituents of the cell. We may also study matter through distance (i.e., structure) morphology, or cytology. Metabolism then is mass considered along a time axis, and structure is mass considered along a distance axis. These relations may be represented as in figure 1 for two particular forms of matter, DNA and RNA. For convenience we set our point of origin in the nucleus. Thus, for example, DNA on the

distance axis will drop off rather sharply as we go away from the nucleus; if we study DNA through time we have the familiar course of DNA synthesis more or less abruptly increasing in mass twofold. RNA is assumed to be broadly distributed throughout the cytoplasm and in the nucleolus and to be synthesized relatively steadily.

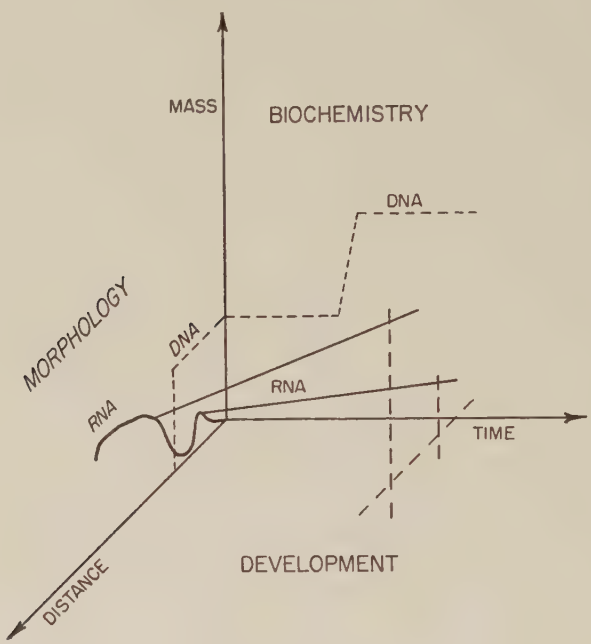

Figure 1

Any component of living matter can be studied in this way. Of course, we usually are obliged to take our sample for chemical analysis as some compromise integrated over a considerable region of distance — as in a tissue mince. More and more cytochemists are trying to do this precisely, studying what is happening locally in distance but often with only the crudest time axis. This way of looking at it makes it easy to see that we need to fill in some precise observations between the axes.

We may consider a chain of biochemical conversions, $C \rightarrow D \rightarrow E \rightarrow F$, and so on in the cell as a steady state in which one chemical component of the sequence may be in a very large amount (as D and F shown here), others in a very small amount, and so on.

$$\rightarrow C \rightleftharpoons \mathbf{D} \rightleftharpoons E \rightleftharpoons \mathbf{F} \rightleftharpoons G \rightleftharpoons H \rightleftharpoons$$

Those that occur in large amounts are the ones that we analyze and consider characteristic of cells. Yet the minor components that turn over so rapidly (the enzymes for the conversion of which are present in large amounts or are highly active) can be thought of in some ways as the most important components. So if we do not find something in the cell, we know that either it is very important or not important at all!

The morphologist and the biochemist have been in the main studying these major components. Isotope methods allow us to study turnover and even to discover the minor constituents and to find the pathways. What I would like to emphasize is that what is characteristic of an organism is not so much the maximally accumulated components in the steady state but the metabolic pathways. The pathway really defines the organism and its behavior along the time axis.

Some substance that is present in the organism in more or less high concentration, when considered on the distance axis, is likely to be localized. For structural components, the rate of change of mass along the distance axis is very abrupt. There is a specific concentration increment that rises very steeply at some place and then drops abruptly in a sort of rectangular shaped curve. This would be typical for what we used to call the characteristic components of organisms.

Now consideration of the flow-state system would remind us that many of the important components of cells are soluble substances. Many of them are present in small concentration, but even those present in large concentration may exist in diffusion gradients. I would like to stress that the concentration gradients of the less-stable, more-soluble substances of the cell are just as characteristic descriptions of the chemical

architecture of the cell or mass of cells as those we have tended to emphasize in either biochemistry or morphology.

It is increasingly possible to consider the time rate of change of matter in different parts of cells. By dealing with matter along both the time and the distance axis and in the vast region in between — not being just morphologists or just biochemists — we will find differentiation less mysterious.

The metabolic gradients are just the factors that are able to change as an embryo develops, changing from one region and time to another, and they may be just as valid and significant structural components of the cells as any macromolecule or membrane with a rectangular distribution curve.

In fact, the lessons of biochemistry suggest that we may think of two kinds of information, the genetic kind, or information storage, and the kind that comes from the impetus of a moving system in an active state. The latter may be described not only in gradients but also in terms of the concept of the primer. The very polysaccharides that Dr. Heidelberger has been talking about have often been used as examples of the way in which the presence of a given construction of matter can lead to more of the same. This is not quite what they illustrate. Given an α-amylose chain of glucoses, the right enzymes can make more α-amylose with a straight chain of newly added glucose residues. This is sometimes mistakenly thought of as the priming of a specific synthesis by a specific preexisting structure. The fact is, however, a glycogen primer, with multiple branches every three or so glucoses, can cause the same enzyme still to add straight chains, merely hanging them on the preexisting end glucose residues. So a product with the specificity determined by the enzyme is still being made and the primer is simply a frame on which to hang it. Furthermore, if only one branch is present, so that a branching enzyme will not be prevented from combining, a branching system on a straight chain can be built very successfully.

This is one kind of priming. This is priming in which the enzyme has only one requirement: a terminal glucose residue

that is far enough away from the other end of the chain or near enough to a branch upon which it proceeds to build in its own fashion. There seems to be another kind of primer turning up; it looks as though the polynucleotide syntheses of S. Ochoa and A. Kornberg are priming systems, too. In these, synthesis proceeds in a manner modified to some extent by the type of structure that exists not only at the end but also far back along the chain. It seems as though the enzyme is asking not merely, "What is on the end; where can I hook something on?" but "What kind of a system do you have?"

The *nuclear* information systems may be of this type because they seem to build entities that can induce the building of other similar systems. The other kind, the non-nuclear ones (which for simplicity we may call the *unclear* mechanisms) carry information in active form and pile it up in gradients, and it is these systems whose principal message is readable in its available end groups or other measure of active status.

Perhaps, therefore, we should also look in systems a little above the molecular level for signs of priming. In these cytoplasmic systems, we might hope to see that the existence of a particular kind of structure may be a required precondition for further production of more of that same structure. For example, I could imagine a membrane, a layer of molecules, flattened out in some kind of sheet, that might uniquely determine extension of such a sheet. The mere existence of such a layer or other special aggregate of oriented molecules could guarantee that more such molecules would also be laid down in the same orientation, and its absence might make such arrangement improbable.

We all know of the chloroplast and other cytoplasmic organelles that are to some extent self-determining. The question might be asked whether such things as membranes and cell walls are similar. Specifically, we might ask whether the popular protoplasts of microorganisms can be made so free of cell wall that, although viable, they find it impossible to continue synthesis of cell wall material any longer.

In summary, the mobile constituents of protoplasm deserve more consideration, for their subtle gradients and arrangements constitute an important part of the order of the cell. Furthermore, the active ends of molecules and molecular aggregates may furnish the orienting influences by which cytoplasmic self-determining systems are propagated along existing lines, in contrast with true genetic determination involving large specific regions of molecular structure.

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CELL POPULATION DYNAMICS AND SOMATIC CHANGE[1]

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THREE FIGURES

Many investigators of microbial systems, particularly those who entered experimental microbiology via general biology, regard their studies also as useful models of developmental systems (e.g., Ephrussi, '53; Sonneborn, '54; Sussman and Sussman, '56; Lederberg and Lederberg, '56; Spiegelman, '45; Braun, '52). Justifications for drawing certain parallels between microbial variation and somatic variation have been expressed so frequently that they do not require restating here. At the same time it remains wise to recognize the limitations of such comparisons, imposed, for example, by the frequent absence of structural and polarity factors in microbial cell populations, as compared to the role of these factors in changes of somatic cell populations (see Weiss, '53; Rose, '57), and by the differences in ploidy that seem to exist between many microbial cell types and most somatic cells. Despite the existence of such obvious differences, there seems to be general agreement that microbial systems can be useful guides to the recognition of the nature and role of many of the factors contributing to somatic change. This is particularly true for problems concerned with changes in somatic cell populations and with interactions of somatic cell types since, with no imputation as to the mechanism responsible for the origin of new cell types, we can compare a number of

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known and suspected phenomena in somatic cell populations with various known phenomena in microbial populations. The usefulness of such comparisons can be illustrated by parallels between information derived from microbial population studies and certain mechanisms independently uncovered in somatic cell population systems, such as selective inhibitory effects of metabolic products observed in bacterial population studies (see Braun, '52) and the corresponding selective inhibition of somatic cells (see Rose, '52, '55, '57). Other observations on variations in somatic cell populations in, e.g., mammalian cell cultures (Puck and Fisher, '56; Puck *et al.*, '57; Haff and Swim, '57) and ascites cell populations (Klein, '55; Klein and Klein, '56; Hauschka *et al.*, '56; Gross *et al.*, '57; Potter and Law, '57), clearly indicate that at least certain somatic changes are identical in manifestation, and possibly cause, to population changes in microbial systems where selective establishment of spontaneously arising variants represents a major mechanism responsible for population changes. These striking parallels are further supported by the similarity of metabolic differences among certain somatic cell types (e.g., Warburg, '56) to the metabolic differences among certain closely related, yet genetically distinct, microbial cell types (e.g., Altenbern *et al.*, '57; Gause, '58). Above all, anyone who has studied microbial population changes, particularly changes involving progressive establishment of different spontaneously occurring antigenic mutants in bacterial populations, cannot help but be impressed with the manner in which competitive cellular interactions can result in a striking regularity of cellular population shifts, resembling in many respects processes of differentiation in higher organisms.

Results of many studies with microorganisms (e.g., Ryan, '53; Bunting, '46) could be mentioned in a discussion designed to suggest mechanisms and factors that may contribute to changes in somatic cell populations. I shall, however, discuss only a few observations from studies with which I have been connected personally, and that have been concerned prin-

cipally with population changes in cultures of *Brucella* species and of *Diplococcus pneumoniae.*

SOME GENERAL ASPECTS OF THE BACTERIAL POPULATION SYSTEM

Most of our bacterial population studies have been concerned with identification of the nature and role of naturally occurring factors that control the establishment of spontaneously arising variant cells differing in antigenic characteristics. These antigenic variants usually also display a simultaneous alteration of virulence and colonial characteristics. Population changes involving such variants were studied in liquid complex or defined synthetic media contained in test tubes. In these closed culture systems, selective forces rather than rate of occurrence of new cell types are primarily responsible for the rate and extent of population changes. The particular mutant cell types with which we have been concerned arise spontaneously at about 1×10^{-7}/bacterium per division cycle. Since our standard inocula contain 1×10^{7} cells/ml of culture, mutant cell types are usually available for selection at a very early culture stage.[2] In some cases, a known number of variant cells were introduced with the inoculum. The rate and extent of population changes involving establishment of the cell types with which we have been concerned are easily ascertained because, upon subcultivation of culture samples on solid media, cells of different antigenic type give rise to different types of colonies. The latter property is responsible for the designations of these cell types as smooth (S), rough (R), and mucoid (M). Population changes

[2] Again, it should be stressed that mutational origin of variants studied in these bacterial systems should not preclude the usefulness of comparing their fate with phenomena that may play a role in somatic cell variation, in which, as has been discussed in preceding papers of this symposium, the origin of new cell types may be attributed to diverse causes. Our main interest in the bacterial studies was focused on interactions among diverse cell types rather than their mode of origin. We assume that the competitive interactions of our physiologically distinct bacterial cell types may reflect the mode of certain interactions occurring among other distinct cell types, regardless of their mode of origin.

involving these variants seem particularly appropriate for comparisons with somatic cell systems, since under most culture conditions, the various antigenic cell types display only relative rather than absolute differences in survival values. As a result, genetically and physiologically distinct cell types can coexist in our bacterial cultures. At any given period in the culture's history, however, a given cell type tends to attain a selective value superior to those of other types under the existing environmental conditions and may thus become predominant.

We uncovered some ways in which metabolic activities of the cells themselves can create specific selective conditions that will interfere with the continued propagation of the parental cell type and lead to the creation of environmental conditions favoring the establishment of other cell types. In our closed culture systems, alterations in the environment attributable to metabolic activities of the parental cell type occur quite rapidly, thus leading to altered survival values of the initial cell type in relation to newly arising types. Details of growth dynamics and population changes in these culture systems, including a description of some factors responsible for the attainment of population and selection pressures need no reiteration here (see Braun, '53). However, some points that seem to deserve particular attention in relation to problems that may also play a role in somatic change will be summarized.

SUGGESTIVE MODELS

Constancy of rate and extent of population changes. In any large series of independent *Brucella* cultures initiated with bacteria derived from the same clone, replicate cultures tend to display an almost identical percentage of antigenically different cell types after a given period of cultural growth (Braun, '46). For example, each one of a series of cultures initiated with S cells of *Brucella abortus* may display 25–28% R cells after 5 days of incubation. This is obviously owing to the interactions of relatively constant rates of occurrence

of altered types and constancy of selective influences at and after occurrence of the new cell type. Constancy of rate and extent of cellular population changes, such as one also observes in developmental differentiation, are therefore possible even where new cell types arise spontaneously and undirected.

Effects of metabolites. Since growth of the initial cell type causes specific modifications of the environment, further regulation of these population events is achieved by the fact that at any period of culture growth, only one of many altered cell types present or arising spontaneously will attain a superior selection value. Thus it has been demonstrated in the *Brucella* studies (see Braun, '52, '53, for details and lists of references) that, when oxygen supply is limited, D-alanine accumulates in the growth medium as a result of the metabolic activity of parental S cells, thereby creating conditions that inhibit continued multiplication of alanine-sensitive S cells. However, certain spontaneously arising non-S cells are significantly more resistant to the effects of D-alanine and thus can establish themselves progressively in these populations. These types also produce alanine and thus contribute to progressively higher alanine concentrations in the culture medium, which eventually reaches levels inhibitory even for this relatively alanine-resistant antigenic cell type. Another cell type with even greater D-alanine resistance, and usually also of different antigenicity, then begins to establish itself. This chain of events can continue and will involve the progressive establishment of variant types more and more resistant to alanine and eventually even alanine-dependent variants (Goodlow *et al.*, '50).

Apart from the apparent regulation introduced into cell population changes by the progressive accumulation of these selective metabolic factors, another phenomenon of interest to problems of somatic change became apparent in these studies. In the course of the sequential establishment of mutants more and more resistant to alanine, some, called S′, occurred that were phenotypically identical to their S-type progenitors yet differed in that they were highly alanine resistant. Therefore,

what upon cursory inspection would have been regarded as the establishment of a back mutant, i.e., an S → R → S change, really involved establishment of an entirely different S type — a change from S → R →S′. It is important that, in the absence of increased alanine levels, the S′ type does not grow so well as its R or S progenitors; it thus represents a cell type that under our culture conditions attains a high survival value only in aging cultures. In a most interesting study on changes within mammalian tissue cultures, Westwood and Titmuss ('57) observed what appeared to be reverse changes, as indicated by cellular morphology and growth rates. This prompted them to abandon their previous interpretation that these changes may be based on principles of mutation and selection. In view of the just-mentioned bacterial data, however, one wonders whether what they regarded as a reverse change may not in reality represent a new cell type that merely mimics a part of the phenotype of a prior cell type.

Influences of substrate and prior changes. Intercellular competition as a regulator of sequential establishment of specific new cell types, from among the many that may occur, can attain even further specificity through (1) the influence of the parental cells' nutritional substrate and (2) the history of the cells' past states (genotypic history of bacteria). For the first phenomenon, effects of different nitrogen sources on the direction of population changes in *Brucella* cultures (Goodlow *et al.,* '52) remain an intriguing example. Mucoid mutants became established in cultures of *Brucella suis* containing L-asparagine as the sole nitrogen source (table 1). At the same time, these cultures accumulated valine but not alanine. The accumulating valine inhibited growth of the parental type, which did produce this amino acid. Spontaneously arising mucoid antigenic mutants were, however, more resistant to the inhibitory influences of valine and therefore established themselves in these cultures. In contrast, rough mutant types established themselves in cultures initiated with D-asparagine as the sole nitrogen source. In these cultures, alanine but not valine accumulated and the rough mutant type that subse-

quently established itself was more resistant to the inhibitory effects of alanine than the parental smooth type cells. We can thus see that the nutrient substrate can control the type of metabolite that accumulates in a closed culture system, and this particular type of metabolite in turn will provide a maximum survival value for specific spontaneously arising mutant cell types. In a way, then, the parental type actually can have a regulating influence on future population changes, determining which *one* of the many spontaneously arising cell types will predominate in a particular population. The determining factor seems to be the particular metabolite produced by the parental cells that eventually will attain concentrations limiting to further propagation of parent cells but not limiting for propagation of specific variant type cells.

TABLE 1

Population changes and metabolism in originally S Brucella suis cultures grown in synthetic medium with D- or L-asparagine as the sole nitrogen source
(From Goodlow *et al.*, '52)

FORM OF ASPARAGINE	PERCENTAGE AND TYPE OF MUTANT CELLS PRESENT ON THE DAYS INDICATED					AMINO ACID METABOLITES IN CULTURE FILTRATE
	5th	8th	12th	16th	20th	
L	0	0	42 M	52 M	61 M	Valine
D	16 R [a]	55 R	84 R	90 R	97 R	Alanine

[a] R = rough; M = mucoid.

How the history of the prior states of the parental cells (which at least in the case of bacterial populations means prior genotypic alterations) can contribute to the specific direction of population changes can be illustrated by some of our observations with bacteria (Kraft and Braun, '54). For example, in a given environment, chloramphenicol-resistant *Brucella* mutants could not be isolated from a certain sensitive strain but could easily be recovered from a crystal violet-resistant mutant of the parental strain. This altered recoverability of the resistant cell type was attributable neither to a cross resistance of crystal violet-resistant cells to chloramphenicol nor to an increased rate of mutation to

chloramphenicol resistance among the crystal violet-resistant cells. Thus the increased survival value of the chloramphenicol-resistant cell type in relation to its parental crystal violet-resistant type permitted its recovery, whereas a chloramphenicol-resistant type arising in the crystal violet-sensitive population had little or no survival value in competition with its parent type. This was confirmed with the aid of the replica plating technique, which clearly demonstrated that the parental cell type can indeed inhibit multiplication of mutant cell types to such an extent that the latter, although they arise at constant rates, fail to go through more than a few cell divisions in the presence of the parental cell type. Genotypic changes of the parental cells, or physiological changes after alteration of environmental conditions, completely altered the relationships between parent and variant cells. The same studies also confirmed frequently reported observations (e.g., Byrson, '50; Bertani, '51; Grigg, '52; Saz and Eagle, '53) that the extent of inhibition exerted by parental cells on mutant cells may be proportional to the density of the parental population. It was further demonstrated that this parental inhibition can be absent in young cultures, thus permitting growth of variant cells artificially introduced at that time, but may increase in the course of multiplication of parental cells, thereby interfering with growth of variants that arise spontaneously at a later stage in the population's growth.

Interactions of cell types. We have now moved from a consideration of inhibitory effects produced by the parental cells against themselves to cases in which such inhibition is directed toward the variant cell type. This, of course, does not exhaust the already uncovered possible types of interactions of cell types, some of which are indicated in table 2.

Returning to the D-alanine inhibition in *Brucella* cultures, I shall refer briefly to observations (Braun *et al.,* '51) that illustrate how the fate of a given cell type may be entirely different, depending on the percentage of another cell type present in the population. Table 3 briefly summarizes the

nature of a phenomenon observed in mixed S and R cultures of a number of bacterial species; namely, attainment of an apparent equilibrium between the two cell types after a period of population growth *independent* of the initial ratio between the two cell types at the start of the population's growth. In

TABLE 2

Some types of interactions between parental and variant cells

CONDITION	PRODUCED BY		AFFECTING		EXAMPLE
	Parental cells	Variant cells	Parental cells	Variant cells	
Inhibitory metabolite	+	—	+	—	Goodlow *et al.*, '50
	+	—	—	+	Saz *et al.*, '53
	—	+	+	—	Cavalli-Sforza and Lederberg, '53
	+ in conjunction with	+	+	—	Braun *et al.*, '57
Depletion of a critical growth factor	+	—	+	—	Ryan, '53
	+	—	—	+	
	—	+	+	—	

TABLE 3

Percentage of R types in 10-day-old broth cultures of Brucella abortus, strain 1², inoculated with various mixtures of S and R type cells

(From Braun, '52)

PERCENTAGE R	AVERAGE RESULTS OBTAINED ON THREE INDEPENDENT CULTURES STARTED WITH SIMILAR INOCULA					
In inoculum	0	1	21	52	78	100
In 10-day-old cultures	18	17	20	21	19	100

the case of S and R cells of *Brucella,* the phenomenon became easy to explain when it was established that S and R cells differ not only in their sensitivity to the inhibitory effects of D-alanine but also in their rate of production of this metabolite. Thus, in mixed S + R cultures initiated with a high percentage of alanine-sensitive S cells, capable of producing

alanine at a high rate, cultural conditions are soon reached favoring selective multiplication of alanine-resistant R cells. These cells, therefore, will increase in cultures started with a high percentage of S cells. R cells produce alanine slowly and, therefore, in cultures initiated with a high percentage of R cells, alanine accumulates only slowly. Since S cells multiply more rapidly than R cells in the presence of low alanine levels, S cells have an initial selective advantage in these cultures started with a high percentage of R cells. The fate of a given cell type can thus be altered to such an extent by the presence of another cell type that its selective value, in the presence of a critical percentage of another cell type, can become actually opposite to that existing in the absence of this second cell type. Similar interactions are probably responsible for a variety of threshold effects, where under standard environmental conditions, certain cell types, though they arise spontaneously at low frequencies or have been added in small numbers, will fail to establish themselves. However, when added beyond a critical threshold concentration, such cells will establish themselves progressively; e.g., R → S changes in *Brucella* cultures: under ordinary laboratory conditions, initially 100% R (avirulent) cultures never display population changes to S (virulent). This is not due, as once believed (Braun, '52), to excessively low R → S mutation rates, because it can be shown that *in vivo* as well as under recently recognized specific environmental conditions (to be mentioned later), R → S population changes do occur in initially 100% R cultures. But, under the usual *in vitro* conditions, S cells do not attain a selective advantage in predominantly R populations, unless their percentage in young cultures exceeds 5% of the population (table 3). Similar threshold effects have been noted in studies with somatic cells, e.g., with ascites cell populations (Klein, '55) and in cases where the take of cells transplanted into hamsters depends on a minimum cell mass, which differs depending on whether the transplant was obtained from originally normal or malignant tissue culture cell lines (Foley, '58). Critical dependence of the selective value

of many cell types on the proportion of other cell types present naturally complicates the reliability and interpretation of data from certain reconstruction experiments with artificial mixtures. This can be particularly true when an experiment in which comparable proportions of two cell types are used is supposed to reflect the fate of cells arising at very low frequencies under natural conditions. Potential pitfalls associated with deductions based on many types of reconstruction experiments have been stressed repeatedly in conjunction with studies on microbial systems (e.g., Braun, '53; Lederberg, '56) but should apply equally to studies with somatic cells. For example, this may represent one of the complicating factors in the studies by Westwood and Titmuss ('57), in which a simple mutational interpretation of population changes observed in their tissue cultures was rejected on the basis of selective values detected in reconstruction experiments involving diploid and polyploid cells. One wonders whether their reconstruction experiments truly reflected natural competitive conditions.

Naturally occurring selective factors. So far, amino acids have been referred to primarily as an example of a naturally occurring selective factor shown to contribute to the outcome of competitive cellular interactions in many different types of bacterial populations (see Braun, '53). A similar significance of amino acids for changes in somatic cell populations may be suspected and might possibly be indicated by the finding that various amino acid analogs have teratogenic effects on chick embryos (Herrmann, '53). However, amino acids represent only one group among the many recognized naturally occurring substances, as well as conditions, that exert selective effects on cellular population changes. The influence of gaseous environment is a particularly effective selective environmental condition. Oxygen and carbon dioxide tensions have a decisive effect on changes in microbial and somatic cell populations (e.g., Braun *et al.*, '56; Altenbern *et al.*, '57; Spratt, '49; Trinkaus and Drake, '56). In fact, the dominant influence of oxygen availability (or of pCO_2 as sug-

gested by Loomis, '57) over the effects of other selective agents, including those of D-alanine in *Brucella* populations, has led to some doubts regarding the causal significance of some of the previously discussed amino acid effects (Altenbern, Ginoza, and Williams, '57; Altenbern *et al.*, '57). For various reasons, I cannot concur with this latter conclusion. I shall, however, refer briefly to one observation with *Brucella* populations that will illustrate the significance of oxygen availability and may serve as a model for illustrating the dependence of the fate of certain cell types on their spatial location [referred to by Weiss ('53) as "field effects"]. It has been known for a very long time that presence of homologous antisera in bacterial cultures will enhance population changes. At least for S → R population changes, it can now be demonstrated that this phenomenon, i.e., enhanced population changes in the presence of S antisera, is not attributable to any direct inhibitory effect of antibodies on S cells, but to anoxic conditions that result secondarily from antibody-antigen interactions (Braun *et al.*, '56). Thus addition of the antiserum to liquid S cultures causes the parental cells, which are usually dispersed through the medium, to agglutinate and settle to the bottom of the culture vessel. Here their rate of growth is significantly suppressed owing to lack of oxygen. However, because of differences in their terminal respiration (Altenbern *et al.*, '57), possibly at the cytochrome level, spontaneously arising R mutants are far less dependent on oxygen tension and will rapidly outgrow their S parents under these conditions. When the agglutinated S cells are kept in suspension by agitation, or when the distance between the bottom of the culture vessel and the liquid-air interphase is decreased, no accelerated population changes occur even in the presence of very high concentrations of antisera. Certain other selective effects of sera and antisera on bacterial population changes, not directly related to oxygen availability, also occur (see Braun, '49); these serum effects will not be discussed here although they may be relevant to some considerations that have been expressed, e.g., by Tyler ('57), in regard

to the role of antibody in developmental differentiation of higher organisms.

The selective effects of oxygen availability on bacterial population changes, just discussed, are paralleled by known and suspected effects of oxygen on variation of somatic cells. Probably the most famous example is Warburg's concept ('56) of the influence of oxygen deprivation on the origin and selective multiplication of cancer cells. To an analyst of bacterial population changes, the selective effects of anaerobiosis are beyond question, but the supposed directed and permanent degradation of the cells' aerobic mechanisms under anaerobic conditions still seems open to discussion and convincing experimental support. Lindegren and Hino's data ('57) on apparent transformations of respiratory-sufficient (AER) yeast cells into respiratory-deficient (aer) cells under the influence of anaerobic conditions, purporting to support Warburg's conclusions regarding the capability of anaerobiosis to transform individual cells, fail to be convincing. These investigators did not have truly adequate controls, i.e., data on the growth of added aer cells in the presence of AER cells, which would exclude possible explanations based on selective effects.

As a final example of the role of naturally occurring factors in the control of cellular population changes, our studies of the effects of deoxyribonucleic acid (DNA) breakdown products may be cited. The basic observation was again made with *Brucella* populations and demonstrated that R → S population changes, which ordinarily do not occur under laboratory conditions, can be promoted with amazing regularity when DNA + DNase are added to the culture medium (Braun and Whallon, '54). This effect was independent of the source of DNA, did not involve any detectable mutagenic effects, and could not be duplicated with the aid of any known breakdown product of DNA (except that kinetin produced somewhat similar effects; see Braun, '57). Subsequent efforts revealed that these unique selective effects of DNA digests were not restricted to *Brucella*, but occurred with every one of

several bacterial species that has so far been tested. It also became evident that, despite the similarity in end results in these various bacterial species (in all of which a promotion of R → S population changes occurred), two entirely different types of mechanisms can be responsible for the observed unusual R → S population changes. The first mechanism has been elucidated in studies with *Brucella* populations, and the second has been identified in more recent studies with pneumococci carried out by W. Firshein in our laboratory. The essential features of these two mechanisms are indicated in figure 1. In *Brucella* populations, the primary breakdown

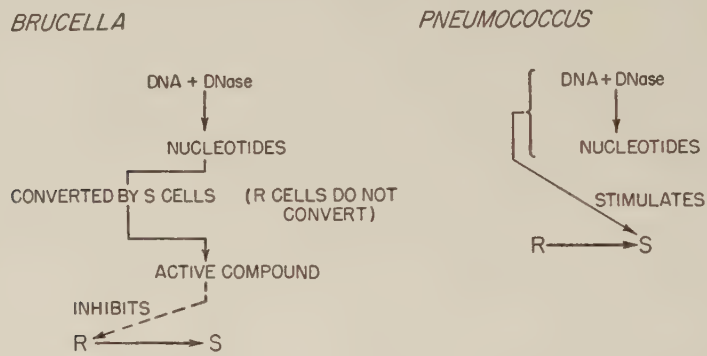

Fig. 1 Mode of action of DNA + DNase (DD) on *Brucella* and *Pneumococcus* populations.

products of DNA have no selective effects. However, S cells arising spontaneously in initially R (or in other non-S) populations convert a DNA breakdown product of relatively large molecular size into a compound that selectively inhibits the growth of non-S cells without interfering with the multiplication of S cells. In this case, therefore, the selective advantage of S cells is attributable to a selective *inhibition* of parental non-S cells. In contrast, in pneumococcal populations, the presence of DNA digests produces a selective *stimulation* of the growth of S cells arising in, or added to, initially R populations. Additional supplementation of DNA + DNase-containing cultures with mixtures of deoxynucleosides, deoxynu-

cleotides, and nucleoside diphosphates results in an even greater stimulation of growth rates of S cells and thus even more rapid R → S population changes (table 4). It is noteworthy that these supplemental effects seem dependent on the presence of all four deoxynucleotides or deoxynucleosides, respectively; removal of any one of these nucleosides or nucleotides reduces the enhancing effects. The findings suggested that DNA digests and supplementation with the cited DNA intermediates may result in a selective stimulation of DNA synthesis in S cells and a lack of such response to extracellular DNA intermediates in R cells. This interpretation was further supported by Firshein's finding that the acid-insoluble

TABLE 4

Effects of various nucleic acid breakdown products on population changes in BHI-blood broth cultures inoculated with 99.9% S plus 0.01% R pneumococci [a]

(Unpublished data of W. Firshein)

	ADDITIONS			PERCENTAGE S AFTER 48 HOURS
	Deoxyribonucleoside mixture [b]	Deoxyribonucleotide mixture [c]	Ribonucleoside diphosphate mixture [d]	
	μg/ml	*μg/ml*	*μg/ml*	
With DNA (130 μg/ml) plus DNase (25 μg/ml)	—	—	—	4
	—	1600	—	15
	1600	—	—	25
	800	800	—	40
	800	800	100	70
	1600	—	100	23
	—	1600	100	18
	Any of the above combinations without DNA + DNase			1

[a] Data obtained without addition of Mn^{++}. When Mn^{++} is added (> 36 μg/ml), a significant enhancement of selective effects occurs.

[b] Contained equal concentrations of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine.

[c] Contained equal concentrations of deoxyadenylic, deoxyguanylic, deoxycytidylic, and thymidylic acids.

[d] Contained equal concentrations of diphosphates of adenosine, guanosine, cytidine, and uridine.

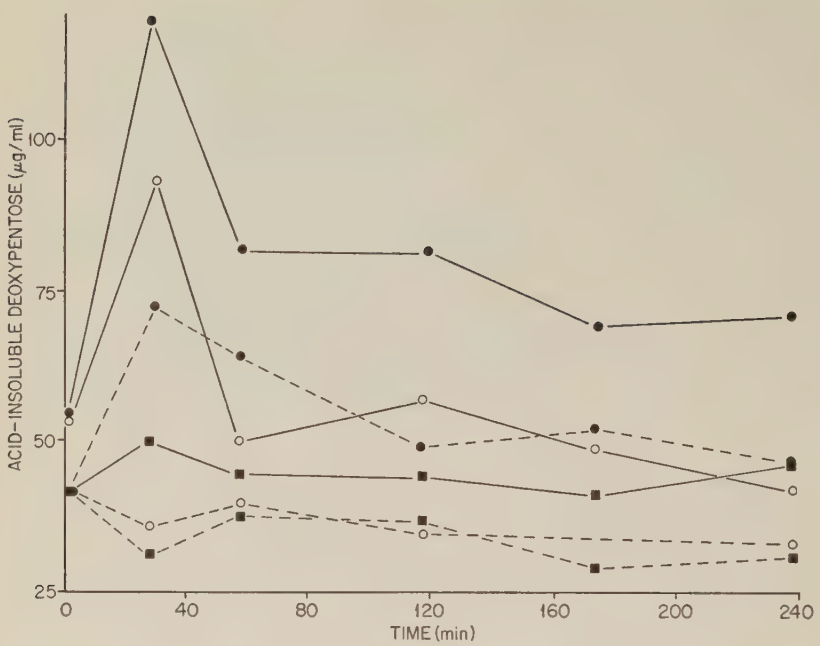

Fig. 2 Acid-insoluble deoxypentose content of resting S and R pneumococci in the presence of various nucleic acid breakdown products. ●, DD + deoxynucleosides + deoxynucleotides + nucleoside diphosphates; ■, control; ○, DD; —, S; - - -, R. The deoxypentose levels were determined by colorimetric analysis (Dische-Stumpf) of hot TCA extracts of the cells (2×10^9/ml). Subsequent determinations for which the Schmidt-Thannhauser method was used yielded comparable differences for the various conditions but also resulted in uniformly lower deoxypentose levels per cell (W. Firshein, unpublished data).

deoxypentose content of resting S-cell suspensions increases significantly and rapidly (fig. 2) under the influence of the supplements listed in table 4. Furthermore, these intracellular increases are directly proportional to the effects of these supplements on growth of S cells (fig. 3) and R → S population changes. It should be stressed that all these effects, on both growing and resting cells, require, as in Kornberg's studies ('57) on cell-free DNA synthesis, the initial presence of small amounts of polymerized DNA. The effects of DNA intermediates on population changes and deoxypentose content of resting cells in the presence of DNA digests, however, were

not directly proportional to the quantity of DNA present in the extracellular environment. This would appear to be in accord with the conclusion that the phenomena just described primarily involve accelerated net DNA synthesis rather than mere intracellular DNA incorporation.

Effects of DNA breakdown products on population changes of both pneumococci and *Brucella abortus* cells have been found to be antagonized by elevated Ca^{++} concentrations (note: Ca^{++} is a potent precipitant of nucleoprotein; see Gross, '57), kinetin riboside, and antisera against DNA (Phillips *et al.*,

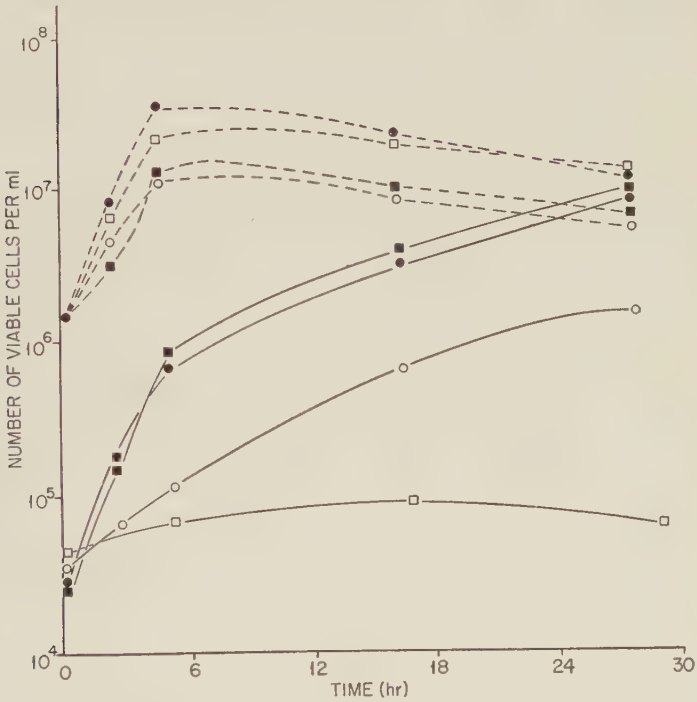

Fig. 3 Viable counts of S and R pneumococci (Type I) in BHI-blood broth in the presence and absence of various nucleic acid breakdown products. ●, DD + deoxynucleosides + deoxynucleotides + nucleoside diphosphates; ■, DD + manganese; ○, DD; □, control; —, S; ----, R. Supplements were added in the concentrations indicated in table 4. Note effects of elevated Mn^{++} levels (55 μg/ml) on enhanced growth of S cells; this corresponds to the enhanced selective effects of Mn^{++} + DNA + DNase in R → S population changes (W. Firshein, unpublished data).

'58). In some unpublished studies (Braun and C. Pootjes), the effects of DNA digests on pneumococci were found to be reversible by salmine, a protamine. Results obtained when salmine alone was added to cultures of *Brucella* and pneumococci reflected, in an inverse manner, the two different selective mechanisms elucidated in studies with DNA digests: salmine promotes R → S changes of *Brucella* populations, since it interferes selectively with the growth of non-S *Brucella* cells. In contrast, salmine promotes S → R changes in pneumococcal populations; this is probably associated with the finding that in pneumococci only the S-type cells respond to extracellular DNA intermediates. These cells, therefore, may be the only ones affected by protamines, which presumably form non-utilizable complexes with DNA and some of its breakdown products.

The possibility of the existence of comparable selective effects of DNA breakdown products on changes in somatic cell populations is suggested by at least three observations: (1) the influence of nucleic acids on developmental differentiation (Brachet, '54; Holtfreter and Hamburger, '55); (2) occurrence of cyclic periods of increased DNase activity (per milligram dry weight), independent of mitotic events, at various stages of embryonic development (Finamore, '55); and (3) enhanced multiplication rates of murine lymphosarcoma ascites cells after injection of the tumor-bearing mice with DNA + DNase; these effects of DNA + DNase, in turn, seemed to be somewhat inhibited by kinetin riboside (Braun and L. Pugh, unpublished data).

Finally, one additional regulatory mechanism affecting the fate of specific cell types is suggested by our recent finding that an antagonist, such as kinetin riboside, will not inhibit the multiplication of S pneumococci in ordinary laboratory media or in mice, but will produce significant selective inhibitory effects in the presence of very small amounts of DNA + DNase (Braun and N. Sakurai, unpublished data). The latter digest ordinarily causes selective growth stimulation of these cell types. Thus this would appear to be another in-

stance in which the extent of selective inhibition is proportional to the rate of synthesis initially occurring in the affected system.

CONCLUSIONS AND SPECULATIONS

The foregoing examples should indicate the extent to which information obtained in microbial population studies can suggest the nature of some factors and mechanisms contributing to interactions among different somatic cell types, regardless of the cells' mode of origin. This brief review concentrated on mechanisms involving spontaneous genetic (i.e., presumably nuclear) alterations and subsequent selection of such altered cell types as one of the major recognized causes of population changes in microorganisms. There are, of course, a number of other mechanisms, such as phenotypic cellular alterations, lysogenic conversions, and the selective establishment of new cell types arising as the result of genetic transfers, that can give rise to temporary or fairly lasting microbial population changes (see Braun and Vogel, '58). Similarly, a variety of different causes are probably responsible for changes in populations of somatic cells; in fact, one great difficulty in assessing causes of somatic changes has been the lack of efficient methods permitting a distinction between somatic cell changes that would correspond to genotypic changes in microorganisms and changes that are merely phenotypic. Embryologists who have recognized the importance of distinguishing between these two classes of alterations have referred to them at times as "modulations," in contrast to processes of "true differentiation" (Weiss, '39, '53), but they have been supplied only recently with techniques (e.g., Puck and Marcus, '55; Puck *et al.*, '57) that promise to permit a better distinction between these two types of alterations in somatic cells.

In view of the demonstrated striking regularity and apparent sequential directedness with which new cell types can establish themselves during microbial population changes based on undirected spontaneous occurrence of a multitude

of different variant cell types, the question may be, and has been, raised: To what extent might comparable mechanisms contribute to developmental differentiation, to so-called "de-differentiation," and to regeneration in higher organisms? It would, of course, seem that a mechanism as useful as natural selection, which has been called upon to explain phenomena ranging all the way from the origin of life (Oparin, '38; Allen, '57), the evolution of species, short-term population changes, intracellular events (Waddington, '48; Weiss, '53; Sonneborn, '54; Holtfreter and Hamburger, '55), to the formation of antibodies (Talmage, '57), may also play an important role in naturally occurring somatic variation. Yet, the involvement of spontaneous mutation-like cellular changes in development frequently has been questioned (e.g., Ephrussi, '53) because it has been generally believed that this would require a rather unlikely process of orderly and directed gene mutation. There are indeed a number of difficulties associated with any generalized application of a concept of mutation and selection to developmental differentiation (see Braun, '52). On the other hand, there are many striking "phenotypic" parallels between the two cell population systems, and there is the demonstration that at least some of the previous suggestions derived from bacterial population studies (Braun, '52) are indeed confirmable by studies in experimental embryology (Rose, '55, '57). Furthermore, there is increasing evidence that progressive spontaneous genotypic changes can become restrictive (e.g., Kraft and Braun, '54; Cavalli-Sforza, '57), and the concept of local genic states (Lederberg and Iino, '56; Sonneborn, '54; Beale, '54) has provided an example of potential mechanisms that could contribute to the spontaneous occurrence at any stage of ontogeny of only a restricted number, rather than an infinite number, of variant cell types on which selective forces can act. Experimental evidence supporting the occurrence of nuclear alterations in developmental differentiation also has been provided in recent studies (see Lehman, '57). Involvement in ontogeny of mechanisms corresponding to mutation and selection there-

fore cannot yet be ruled out. In any event, if we choose to pursue our parallels to microbial population systems one step further, we could anticipate that the selective establishment of a given cell at specific ontogenic stages and at specific sites may also lead to retention of small numbers of other variant cell types as well as of some parental cell types (cell remnants). This possibility raises the following questions:

1. To what extent might differences in the so-called lability (transformability) and fixity of different cell types (Trinkaus, '56; Grobstein, '55) actually reflect differences existing on the population, i.e., tissue level? How often is lability a reflection of heterogeneous cell remnants, whereas fixity reflects cellular homogeneity?

2. To what extent do tumor cell types arise after renewed but limited multiplication of the original cell type caused by release of the normal inhibitory forces preventing such multiplication? Or, as Weiss ('53) has expressed it: to what extent is apparent dedifferentiation really a redifferentiation? Also, to what extent are some carcinogenic events associated, as has been frequently suggested, with multiplication of cell remnants after release of normally existing inhibitions by other cell types? [3]

3. To what extent might the apparent pluropotency of cells in regeneration (Needham, '52; Trinkaus, '56) really reflect a pluropotency of existing heterogeneous cell populations? Does the removal of inhibitory cell masses (Braun, '52; Rose, '55) reinstate the capacity of cell remnants to multiply and undergo the type of changes ordinarily occurring in the embryonic stage?

4. To what extent is the influence of cell mass in experimental embryology (Trinkaus, '56; Grobstein, '55) indicative of a relation between cell numbers and the likelihood for carrying over heterogeneous cell types?

[3] Note that studies on changes in ascites cell populations (Klein, '55) demonstrated that both preexistence of variant cell types and the new occurrence of such cell types can contribute to somatic changes.

5. Does Lillie's well-known evidence for embryonic segregation ('29) possibly represent, in some instances, an inability of isolated variant clones to undergo population changes comparable to those that can occur during coexistence of such clones?

It can be anticipated that, with the aid of some of the recently developed techniques, (see, e.g., Puck and Marcus, '56; Trinkaus and Groves, '55; Gwatkin *et al.,* '57), some definite answers to these and related questions may soon be forthcoming.

In conclusion, permit me to append a plea, which is directed principally to those currently engaged in certain applied areas of somatic variation. As you know, there has been a rapid increase in efforts directed toward identification of potential antitumor agents with the aid of test systems involving cell lines of malignant and normal origin maintained in tissue cultures. Most of these test systems involve cell cultures consisting predominantly of one cell type. To anyone who has seen, in the course of microbial population studies, how different inhibitory effects can be in mixed cultures compared to cultures consisting of one cell type, the present tissue-culture test systems seem at times dangerously oversimplified. These investigators should therefore be urged to develop adequate procedures for assaying the fate of somatic cell types in mixed populations containing known proportions of different cell types. Unless such known mixed population systems, corresponding as closely as possible to those present under natural conditions, are used for the study of environmental agents capable of interfering with the multiplication of undesirable cell types, the information obtained may be comparable to that obtained by an observer who attempts to judge the shape of an object by its shadow at 5 minutes before noon.

OPEN DISCUSSION

EPHRUSSI[4]: I should like to make a very short remark to dispel a slight misconception. From the very beginning of

[4] Boris Ephrussi, University of Paris.

my studies of respiration-deficient mutant yeast, it was very clear to me that there is an obvious, but superficial, analogy between these mutants and cancer cells, and it was a kind of old-fashioned dislike for advertisement that prompted me never to put a word about this in print. This was so much so that some people probably considered that I just did not see the point. A few years ago a representative of Eli Lilly Company said to me, "Don't you see that you are working with cancer cells?" Well, I didn't.

This brings me to Dr. Braun's point about Lindegren's recent publication on this matter: I completely agree with Dr. Braun. I carefully read Lindegren's experiments and they are just not convincing. I may say moreover that in my laboratory some years ago Dr. Morgan Harris, from Berkeley, made a very detailed study of the effect of anaerobiosis on the production of "petites" (this is exactly what Lindegren tried to do), and I think Dr. Morgan Harris has extremely good evidence that there is no effect.

I would also like to dispel a misconception inherent in such work. This is important at this point, because, as you may have seen from a recent article by Gause in *Science,* these respiration-deficient mutants are being used rather widely now in some institutions for screening anticancer drugs. This is all based on Warburg's conception that cancer cells are induced by respiratory poison. I know nothing about cancer cells, but I can tell you about our respiratory mutants. We know several factors that will induce these mutants at concentrations that have absolutely no depressing effect on the actual respiratory act. But there is a certain correlation between the mutagenic action of these agents and their action *not on respiration but on the synthesis of respiratory enzymes.* I think this is an important distinction.

Hotchkiss[5]: I should like to mention one precautionary note that centers around the relatedness of the rough and smooth types. This kind of study, with pneumococcus at least, would have most significance if the smooth and rough strains

[5] R. D. Hotchkiss, The Rockefeller Institute for Medical Research.

were closely related. I think I know that the majority of your experiments were done with stock types rather distant from each other — isolated at widely different times from different hosts, for example — and I would like to know which part was done with close relatives. Anyway, it is well to point out that this is a risk at almost any time in the laboratory. There are only a few ways to find out the nature of a bacterial strain. We really have become so skeptical that, if we want to work with two strains so closely related that their genetic difference is only a single factor or so, we choose strains that have mutated only within recent experience. I would say that the classical smooth A-66 type 3, which I know was used in some of your experiments with smooth pneumococcus, was shown in a very straightforward piece of work by Harriet Ephrussi Taylor to have a metabolic difference in the way of handling lactic acid. There was a colony difference that her eyes were sharp enough to see in the roughs derived from these strains, and they grew faster on a standard medium. I think the accumulation of such differences would make it very difficult to draw conclusions from strains that had been separated any distance.

Braun: I am, of course, completely aware of the dangers inherent in any experiment in which you attempt to mix two cell types that may not only come from different cultures but may also have been isolated as recently as 2 days ago. I have great respect for the rapidity with which changes can occur in rapidly reproducing microbial populations. Let me stress that, in all the *Brucella* studies, we always relied on spontaneously arising mutants to furnish the raw material for selection. There can be absolutely no question here about the relatedness between the variant and the parent cell types. In the studies with pneumococci we had certain difficulties that happen to be partly responsible for the special suitability of pneumococci for transformation studies: many smooth strains have exceedingly low spontaneous rates of mutation to rough. Mr. Firshein, therefore, frequently used mixtures of smooth and rough cells; i.e., he introduced smooth cells

into 99.99% rough populations that came from stock cultures started quite some time ago and that certainly may differ from each other in many genes. However, he has now repeated all the critical experiments with a rough strain of pneumococci that has a sufficiently high spontaneous mutation rate to smooth so that he could start out with rough populations. The results were identical. More recently, he also repeated some of his critical experiments with mixed inocula derived from cultures that you described to him as being more closely related. So far, data collected with these strains proved identical with those of the earlier ones.

In addition, the data collected with pneumococcal and *Brucella* populations have now been found applicable to other bacterial species and have even been shown to apply to certain somatic cell types. I am therefore not worried about the particular problem that you raised.

HABER [6]: Do you think the activity of DNA digests might be caused by traces of kinetin or similar compounds? Is there any evidence that kinetin riboside acts as an antimetabolite of kinetin?

BRAUN: We do not know how kinetin works, but we suspect that the mode of action of kinetin is different from that of DNA digests because we have strains that are resistant to the selective effects of DNA digests but are susceptible to the effects of kinetin, and vice versa. We have given up working with kinetin because, in contrast to the uniform effects of DNA and DNase, which are repeatable day after day and month after month, the effects of kinetin can be irregular. One of the reasons for this is that the effects of kinetin are influenced by high amino acid and trace metal levels, whereas these do not influence the effects of DNA + DNase.

YAMADA [7]: In embryology, we often have a system that subsequently leads to formation of various components arranged in a definite spatial relation. For example, Dr. Hayashi, in our laboratory, put a sample of ribonucleoprotein of the

[6] A. H. Haber, Oak Ridge National Laboratory.

[7] Tuneo Yamada, Nagoya University.

liver on the isolated ectoderm of the amphibian gastrula and found that the cells of the explant in the direct vicinity of the sample differentiated the optic rudiment. Next to it, a forebrain was formed. Cells at the periphery of the explant away from the implanted sample formed lens, nasal placode, and mesenchyme. If no sample was added to the original explant, only epidermis cells were differentiated. Thus, under the influence of the sample of liver ribonucleoprotein, various tissues could be differentiated from the same ectoderm. The spatial relation of the cells with the solid sample seems decisive for the type of tissues they are going to differentiate. This spatial relation is probably caused by the concentration gradient of the agent. Do you have similar phenomena in your field, in which various concentrations of one and the same agent determine various types of cell population?

Braun: I mentioned that, in cases of accumulating metabolic products with selective toxicity, specific cell types find an optimum survival value at a given concentration of that metabolic product.

Gelfant [8]: Since you did not list the controls in your chart, I assume you used DNase with nucleotides other than DNA, DNase with DNA protein, RNA and RNase, etc. to prove the specificity of DNA digests and also the effects of aerobic and anaerobic conditions on the action of DNA and DNase. I would also like to comment on your analogy between use of mixed bacterial cell types in a culture system and a somatic tissue composed of heterogeneous cell types. The different cell types in an organ such as the rat uterus do not all grow in the same way. Although location of different cell types in the uterus in relation to one another may play a role, the different types of cells have individual growth patterns. This might be worth considering when trying to relate somatic conditions to those in your culture system.

Braun: We used all the controls we could think of, including those you mentioned specifically, but we have not studied anaerobic conditions.

[8] Seymour Gelfant, Syracuse University.

STRAUSS [9]: Would you conclude from these results and the fact that the deoxynucleotides alone do not work that the effective product in these transformations is fairly high polymerized polynucleotides?

BRAUN: First I do not think we should use the term "transformation." It is not appropriate in this connection. I can only guess at what is happening here. We do know that, unless polymerized DNA is present, the whole story does not work. Whether DNA acts as a primer the way Kornberg has suggested or whether it furnishes a necessary and hitherto unrecognized breakdown product, I just do not know.

STRAUSS: Will the system work if you treat DNA + DNase for a period of time and then inactivate the DNase and return the mixture to the cells?

BRAUN: Yes, it does. Also, Mr. Firshein fractionated these DNA digests by Bendich's ECTEOLA procedure and isolated a fraction active in the pneumococcal system. It was of relatively small molecular size. Interestingly enough, the fraction containing the precursor of the inhibitory compound active in the *Brucella* system is found in the large molecular fraction, the one that comes off the column last.

In reference to the effects of DNA digest on somatic cells, let me just state that these observations are based on a limited number of experiments and I would much prefer to discuss them in greater detail when we have more evidence. In preliminary experiments, however, we used Gardner's lymphosarcoma in the ascitic form. We inoculated mice with 450 μg of DNA, either from bacterial sources or calf thymus (but most commercially available preparations do not work), and 150 μg of pancreatic DNase. This significantly enhances the rate of multiplication of the tumor cells, as revealed by smears, and a shortening of survival time of the animals. Preliminary observations also indicate that, just as in pneumococcal infection of mice, kinetin riboside alone does not affect multiplication of these lymphosarcoma cells. When mice were treated simultaneously with kinetin riboside *and*

[9] B. S. Strauss, Syracuse University.

small amounts of DNA + DNase, however, kinetin riboside inhibited multiplication of these tumor cells as well as that of pneumococcal cells.

SUSKIND[10]: Can you amplify a bit on the effect of anti-DNA sera on the DNA effect?

BRAUN: With the aid of a 0.5% phenol extraction procedure, we isolated a DNA-protein that was somewhat unique because it contained about 25% protein that cannot be dissociated from the DNA by any chemical means, such as Sevaging (which is amyl alcohol plus chloroform treatment), enzymic hydrolysis, or treatment with detergents or high salt concentrations. When this material is injected into rabbits (this investigation, as I previously stated, was initiated by Dr. Phillips in our laboratory, and is now being continued in cooperation with Dr. Plescia), antisera are produced that give precipitin reactions with highly purified DNA. The reacting antigens are destroyed by DNase but are destroyed fairly slowly. The latter may be one reason for our successful results because it takes 64 hours of DNase treatment before all reacting material completely disappears. Thus it may be that the uniquely bound protein in this DNA-rich material protects the DNA from depolymerization by DNases normally present in all animals.

This DNA incidentally does transform *Brucella,* and our phenol extraction procedure was actually the accidental result of attempts to get transforming DNA from *Brucella* cells. Since many of the strains used were virulent, we wanted to kill them first. So we killed them with 0.5% phenol, shaking them for 48 hours, and then centrifuged and extracted the cells by conventional procedures. We got some DNA but not very much. Subsequently, we discovered that the bulk of the DNA was in the phenol supernatant and possessed the previously mentioned unique properties.

The antisera resulting from the immunization of rabbits with this DNA have many unique properties. For example, these antisera produce typical L. E. bodies in human white

[10] S. R. Suskind, The Johns Hopkins University.

blood cells. You may know that sera of patients suffering from a disease known as lupus erythematosus produce this phenomenon, and recent studies by Holman and Kunkel showed that sera from these patients frequently contain antibodies against DNA. Our rabbit antisera and L. E. sera will antagonize the DNA + DNase effects I described. Occasionally, antisera against bacterial DNA preparations also have shown very weak cross reactions with DNA from foreign sources. For example, the antiserum produced against *Brucella* DNA was found by Dr. Phillips to cross-react weakly with purified calf thymus DNA or with fish sperm DNA, and such cross reactions were enhanced by partial depolymerization of the foreign DNA.

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MELANOTIC TUMORS IN *DROSOPHILA*

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THREE FIGURES

Melanotic tumors in insects have been known for a long time. In *Drosophila,* these tumors have been considered in relation to the problem of cancer, which, in turn, has some connection with somatic variation of genetic origin. Whether that relation exists generally is doubtful. But cases are known in which neoplastic invasion in *Drosophila* is linked to production of melanotic masses. I believe, however, that melanotic tumors should be studied, not only in relation to the cancer problem, but also because they offer an analyzable case of genotypically controlled histological differentiation. In fact, in many stocks we deal with a larval character that can be already complete during the second instar or even earlier (fig. 1). Moreover, the character, especially in its earlier stages, manifests itself in different, completely viable ways. The genotype, then, is not restricted to the usual alternative — lethal-viable — as so often happens in the embryonic characters.

DEVELOPMENT OF MELANOTIC TUMORS

Pseudotumors. Melanotic masses appear during larval, pupal, or adult stage, according to the genotype. They are composed of melanin and some of the cells that produce it. Some tumors appear to be inert masses that apparently cause no harm to the carrier. Tissue culture proved, however, that they contain living cells. Some evidence, taken from selection experiments, even indicates that presence of tumors is of some advantage to the fly. These masses have been called "pseudo-

tumors'' (Barigozzi, '54), for they, lacking cell invasiveness, have almost nothing to do with malignant neoplasms. Many examples are known of stocks that have arisen independently, showing localization of the melanotic masses in particular organs (e.g., fat body and pericardial cells), a loose attachment to some of the viscera contained in the abdomen, or restriction to the head or thorax.

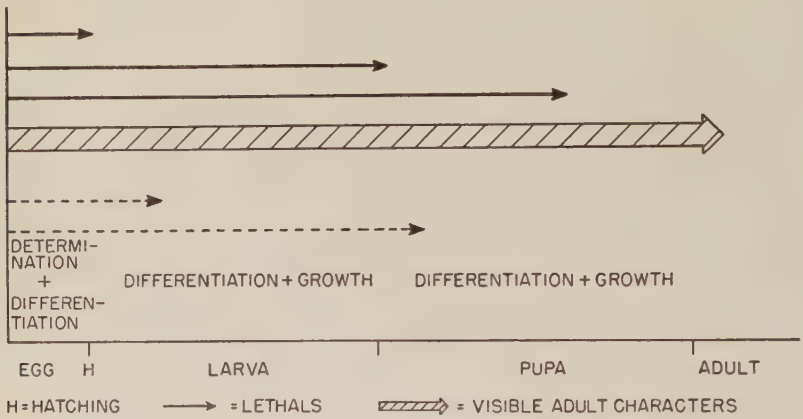

Fig. 1 Scheme of the relation between development and tumor formation. Dotted arrows indicate the development of the tumors in different stocks.

Development of these melanotic masses has been followed in different stocks. Several authors (Oftedal, '53; Kaplan, '55) found that melanin is produced from migrant spindle-like cells. In our department, Castiglioni ('56) used stocks A_2, B_3, C_4, and melanotic e 144 in her research. On the basis of previous and other findings (Castiglioni, '57, and unpublished data), she is now studying the origin of migrant cells. In this research, she compared tumorous with tumorless stocks (Varese, ClB/+; Cy L/Pm; H/Sb Mé). Staining of lymph drops with May-Grünwald-Giemsa shows four types of cells migrating through the larval body cavity: small and mid-sized basophilic cells, large cells with undefined margin (two to four times as large as the mid-sized ones), and cells with crystalloid inclusions. There are also some intermediate

stages. The frequency and total number of these cell types change with the genotype. Countings made on hemolymph can give a wrong estimate of the real frequencies within the lymph gland, because a differential release from the gland for each cell type is possible. On the other hand, no safe classification of cells can be made on sections where individual characters become almost invisible. Nonetheless, their distribution within the gland seems to be random. Only the large cells are involved in production of melanin. In our stocks, tumors can form only when the large cells comprise at least 3% of the total cell number; sometimes the proportion may be at least 15%. The large cells and other cell types differentiate within the lymph gland (sometimes forming multinucleated bodies) during development. These cells multiply mitotically in spite of their complete differentiation.

It is interesting to note that the resting nucleus is slightly polythenic, and that mitotic chromosomes are remarkably large (Barigozzi, '57). Large cells probably do not multiply and perhaps undergo endomitoses. This might explain their size.

Cells can be released from the lymph gland in two different ways. When the gland reaches a certain developmental stage and forms two series of lobes right and left of the aorta (the whole gland was studied; it was prepared in agar after a slight staining with Delafield hematoxylin), some of the cells leave the gland. They migrate either singly through the gland membrane, the gland itself remaining intact (e.g., Varese) or through disintegration of the anterior lobes of the gland (e.g., A_2, melanotic e 144). In stock tu B_3, the anterior lobes detach from the remaining gland and migrate through the body cavity. The large cells, when clumped together, become flat and begin to form melanin, which is deposited intercellularly. Eventually, its amount exceeds that of the cellular substance, giving the impression that the final product is merely a melanin mass.

Melanotic masses can reach a diameter of 0.1 mm. They are frequently much smaller and are visible only at 40 magnifica-

tion. In many genotypes, melanotic clumps are produced comprising only a few cells.

Different developmental mechanisms are at work in other stocks. For example, Kaplan ('55) describes the development of tu-e, where migrant cells (whose origin was not investigated) crowd around the pericardial cells and finally produce pericardial melanizations. Other organs can also melanize in the same stock.

Lethal and invasive tumors. Other melanotic tumors are more or less clearly connected with lethality. In some cases, they resemble the vertebrate cancers. A doubtful case is represented by Raimondi's stock marked by a semidominant mutation *Sine Oculis* ('56). In this stock, only 5.4% of the adults show melanotic tumors, whereas larvae 0.2–2.5 mm long manifest an incidence of 33%; 23% of tumorous larvae die before pupation. Since mortality is also high during embryonic development, it is not clear how melanotic masses cause death.

A more typical case of lethal tumor in which a true invasion of the imaginal discs is brought about by the lymph cells is described by El Shatoury ('55b). In this stock (lethal malignant, l-m), malignancy consists of abnormal proliferation of both the lymph gland and the stomach epithelium. Deposition of melanin should represent a kind of cell reaction when cells are "antagonized" (El Shatoury and Waddington, '57). In this case, the neoplastic tissue is not composed of melanizing cells.

Another case of lethality connected with formation of melanotic masses is found in the stock marked with *multiple wing hairs* a recessive mutant of the third chromosome (Di Pasquale, '52). No linkage identity is demonstrated between melanization and the marker. In this stock, 4.5% of the adults develop tumors but 17.7% of the pupae die. Nearly all these pupae that die (88.8%) show melanotic masses as well as several abnormalities of the head, thorax, and abdomen. The connection between these phenomena is unknown.

DEVELOPMENT OF MELANOTIC TUMORS AND NUCLEAR CONTROL

Melanotic masses are genotypically induced. In cases analyzed only for melanization (i.e., not considering lymph cells and lymph gland), the second chromosome seems to exert a prominent influence (Bridges, Herskowitz, Burdette, Hartung, Ghelelowitch, Stark, and Bridges, Russel, Wilson; for reference, see Barigozzi and Di Pasquale, '56). The third chromosome is sometimes considered a site of modifiers. Stock *tu-er* deserves special consideration (Glass and Plaine, '52). It is a stock where there is one factor in the second chromosome for the production of melanotic pseudotumors and another factor in the third chromosome that acts as a suppressor and whose action can be blocked by different physical (X rays) and chemical means. El Shatoury's *l-m* ('55a) is located on the first chromosome. Other stocks are known, where an action exerted by chromosome IV pair or by two chromosomes simultaneously can be demonstrated.

Stocks A_2, B_3, melanotic e 144, and C_4 were investigated by the following techniques. The three major chromosomes were tested separately for their action by replacing each of them with the corresponding elements of the stock ClB/+; Cy L/Pm; H/Sb Mé. A more-accurate analysis of chromosome II was made by means of recombinants between the tumorous stock and *b cn vg* (3% tumorous) and *cn c px* (practically tumorless). For studies of the corresponding phenotype, recombinants were made isogenic. Manifestation in heterozygous state was studied in the tumorless stock Varese.

The genotypic control of the three main components of the phenotype is as follows: (1) *Large lymph cells* are controlled by chromosomes I and II; nothing can be said of chromosome III. High frequency behaves nearly as recessive. (2) The *state of the lymph gland* is controlled by all three major chromosomes. Intact gland behaves as dominant against disintegrated gland. (3) *Melanization* is controlled nearly exclusively by chromosome II in stocks A_2, B_3, and melanotic e 144; and in C_4, by both the first and second chromosomes.

Melanization is recessive to nonmelanization in A_2, B_3, and in melanotic e 144. In C_4, it behaves as dominant (large cells and gland behavior were not analyzed).

Polygenic factors affecting melanization and the state of the gland are located on the left end of chromosome II in A_2 and on the right end in B_3 (Barigozzi and Di Pasquale, '56). The frequency of large cells is influenced by factors located more proximally on chromosome II. This is shown by tumorless recombinants that show high frequency of large cells but that have intact gland and no melanization.

In summary, production of melanotic masses in the analyzed stocks is the end result, depending on three factors (each multichromosomal and probably polygenic) dealing with three phenomena: (1) release of these cells from the lymph gland into the body (multichromosomal), (2) presence of a rather high percentage of large hemolymph cells (multichromosomal), and (3) production of melanin and its associated changes in cellular morphology (chromosome II, or multichromosomal) (figs. 2, 3).

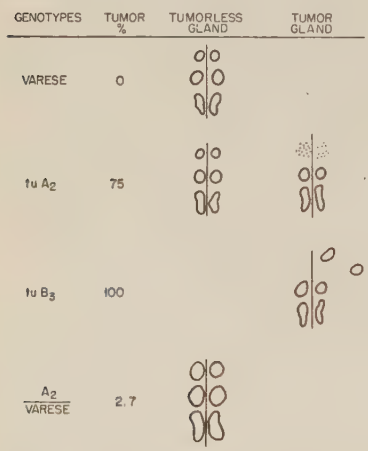

Figure 2

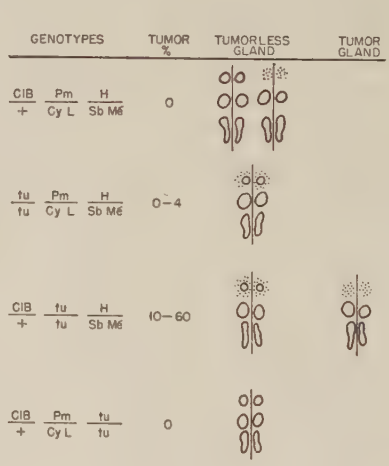

Figure 3

Fig. 2 Scheme of the genotypic effect on tumor frequency and lymph gland: tumorless and tumorous stocks, and heterozygotes.

Fig. 3 Scheme of the genotypic effect on tumor frequency and lymph gland: different genotypes including chromosomes from tumorless stocks.

A different variation range exists for the three steps involved in tumor production. This is demonstrated by a comparison between the genotypes: A_2 (original stock) and ClB/+; A_2/A_2; H/Sb Mé.

In the first genotype, 75% of the flies carry tumors and differ sharply from the 25% that lack them, both in frequency of large cells and state of the gland. In the second genotype, the 60% tumorous and the 40% tumorless flies differ very little, if any, for the mentioned characters. The lesser variability of both large-cell frequency and the state of the gland in ClB/+; A_2/A_2; H/Sb Mé may reasonably be explained partially as a greater stability of chromosomes I and III in this genotype (if compared with A_2 original stock) and partially as a lesser penetrance of the melanization genes. In other instances, the genetic heterogeneity of a stock is demonstrated through selection experiments. Disintegration of the gland is more complete in tumorous than in tumorless larvae. This stock is, to a large extent, heterozygous for melanization genes.

Heterogeneity in genotypes of tumorous stocks is indicated by the following: (1) nearly all so-called tumorous stocks show a very low percentage of tumors and (2) flies collected in the wild are very often tumorous, but produce variable percentages of tumorous offspring, after insemination in nature (Di Pasquale, unpublished).

Now if we take into consideration the stock *tu-er*, which has not yet been investigated with cytological methods, we find that the suppression exerted by chromosome III on chromosome II might be understood as an action on frequency of large cells or on gland behavior, whereas the chromosome II factor might be a gene or a genes system for melanization.

CYTOPLASMIC CONTROL OF MELANOTIC TUMORS

Makino and Kanehisa ('54) and Kanehisa ('54) first detected a cytoplasmic effect in the production of pseudotumors in *D. virilis*. Di Pasquale ('56) demonstrated an extranuclear effect in A_2, B_3, C_4, and D that shows chiefly a nuclear trans-

mission. To demonstrate cytoplasmic activity, we replaced all three major chromosomes of tumorous stock with those of a tumorless one. The donors of cytoplasm were the four tumorous stocks; chromosomes came from ClB/+; Cy L/Pm; H/Sb Mé. The replacement technique was based on transfer of the entire chromosomes. After chromosome replacement, tumors were still present in all lines (two independent lines for each tumorous stock), their frequency ranging from 2.3 to 9.7%.

To demonstrate cytoplasmic activity, we replaced the genome of the B_3 tumorous stock by nine successive backcrosses to the tumorless stock Chieti (0.7%). Since, after chromosome replacement in 15 independent lines, tumor percentage was 5–10, the effect of cytoplasm seemed to be demonstrated. In this case, however, there is no genetic evidence that the cytoplasm is really independent in inducing tumors from the nuclear genotype.

DISCUSSION

The basic character of pseudotumors is the production of melanin by normally differentiated cells, without any abnormal rate in cell multiplication. The tumors (or cancers), on the other hand, are formed by abnormal cells that multiply rapidly and invade other organs. All cases of melanization in *Drosophila* not associated with high rate of cell reproduction and invasion should be considered as different from tumors. This does not deny that there are some similarities between tumors and pseudotumors and that in *Drosophila* true tumors showing melanization can exist. The cases described do show abnormal cell multiplication as well as melanization, but the connections between the two aspects are not clear.

Cell differentiation is one of the most general phenomena in living organisms. It consists in acquiring new specialized and generally irreversible characters during development; differentiated cells derive from an original undifferentiated stage. Examples of this phenomenon are blood cells of vertebrates and the lymph cells of *Drosophila*. In the former,

hematologists have built pedigrees of the different cell types (red and white cells). The lymph cells of *Drosophila* have not been studied so thoroughly. We know only that differentiation into the four cell types described is really complete at the beginning of the third instar, and that mitoses are detectable in the small and in the mid-sized basophilic cells. The frequency variation of the four cell types in the hemolymph poses the problem of how the genotype controls that variation. This could be because of: (1) differential production of cell types in the lymph gland (brought about by different rates of differentiation or proliferation) or (2) differential release of cell types from the lymph gland.

Cells that undergo clumping change their form, becoming thin and flat. After that, they produce melanin. This means a transformation of a small group of differentiated cells into a new stage. This phenomenon poses at least two problems: (1) how to conceive the ability of the big cells to change greatly their form and their function. Since ability to melanize is very common in insects (indeed as a result of the most varied injuries), I am inclined to admit that many cell types of *Drosophila* have the capacity to melanize as a hidden potentiality. We do not know which is the inducer (Castiglioni and Beati, '54). Nothing can be said about those tumorous stocks, where a cell type is particularly involved. (2) The stimulus that causes cells to congregate and produce melanin in one or more regions of the body is subject to different influences. In some stocks localization is stable (fat bodies, head); in others it is changeable. The pattern is certainly greatly influenced by the genotype.

SUMMARY

Melanotic tumors in *Drosophila* either are pseudotumors, and then fail to produce any harm to the carrier, or show some similarities to cancer. In general, melanotic tumors occur in larvae but appear also in the adult.

Melanotic masses (pseudotumors) are the product of migrant cells that differentiate within the lymph gland. A suitable percentage of a peculiar cell type and a structural transformation of the lymph gland are prerequisite for melanization.

All mentioned steps leading to melanization are controlled by different parts of the genotype. The cytoplasm also exerts some influence.

Thus melanotic tumors become an interesting case of somatic variation, genetically analyzable.

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GENETIC APPROACHES TO SOMATIC CELL VARIATION: SUMMARY COMMENT

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Up to this point I would heartily concur with the felicitations that were offered to the organizers of this program, but in one respect I think they might have served us better. We have worked hard in crowded long sessions, and perhaps you deserve something more pleasant and jocular than I am competent to offer.

One thing I will not do is summarize what was said, since that will be found in the papers themselves. I suspect that my intended function is to summarize what was *not* said.

I left the last session before I was able to discover what the molecular basis of somatic variation is, and that was only so I would have an opportunity to collect these thoughts. Perhaps there will be some opportunity in the rebuttal to the discussion, or the discussion of the rebuttal, to make up for it.

I think we should be grateful to Dr. Stern for opening the session with his summary of very useful and important facts concerning processes of variation in somatic cells. An understanding of the mechanical history of the chromosome is, of course, basic to any of the further speculations that we may like to build, and it is some comfort, indeed, to know that there is rather concrete evidence for such processes as mitotic crossing over and endomitosis that we might like to invoke.

He also, I was glad to note, pointed out some difficulties. It is still rather hard to understand how precise triploid complements can be gotten by any simple somatic process. I think it may be necessary to give more attention to the problems of genome segregation that the late Professor C. L. Huskins, of Wisconsin, was so interested in, and which are paralleled in the enigma of the ciliate macronucleus.

[1] After Feb. 1, 1959, address Stanford University, California.

I am still rather puzzled why biologists show such a strong antisexual bias in the consideration of somatic cells. On the other hand, I think I would have been the first to ridicule the fantasy that viruses might carry bits of genetic material from one cell to another in a transductive process, and yet suggestions of this kind seem to be accepted with great gullibility. Projections for future experimentation on somatic cells have invoked transductive phenomena almost to the exclusion of mating. After all, if we combine Stern's discussion with Hauschka's, we will see that every single one of the unit processes needed for the technical handling of mating has been documented in somatic cells. True, they have not been serially documented on a given set of cells under experimental control. But we have reports of the fusion of somatic cells. We know that nuclei of binucleate cells can fuse, if only by coalescence of the spindles at the next mitosis. We know we can have somatic segregation as well as mitotic crossing over. Fifteen years ago we had a much more negative outlook with regard to the possibility of Mendelian analysis with such organisms as bacteria, viruses, and *Penicillium* than we now have for somatic cells.

Ephrussi initiated a provocative discussion on the classification of genetic phenomena, which is of some importance for any attempt to relate the facts of cellular differentiation to the framework of genetic theory. In the past, we have contrasted chromosomal versus extrachromosomal. Reporting on a recent conference on cytoplasmic heredity held at Gif, he suggests that this contrast may be misdirected. He would define hereditary phenomena as either "genetic" (in a stricter sense) or "epigenetic," according to whether the information is structural or based on some sort of dynamic flux equilibrium. In the English language, at least, this particular choice of terms may be confusing if only because "epigenetic" is already widely current in a different sense, e.g., in Waddington's book "The Epigenetics of Birds." The proposal is so provocative, however, that we ought not to dwell merely on its verbal aspects.

As has been pointed out, we can now begin to ask very concrete questions on the *chemical* basis of genetic differentials. We can no longer doubt the role of nucleic acids in this context, and rather than debate it any further I think we can define a category of genetic information as being "nucleic"; that is, depending on the *sequence of nucleotides* in a nucleic acid. By contrast, "epinucleic" information is expressed in another form, e.g., as an aspect of nucleic acid configuration other than nucleotide sequence or in polypeptide or polyamine adjuncts to the polynucleotide. We also have extranucleic information in molecules or reaction cycles not directly connected with nucleic acid. In accord with Ephrussi's suggestion, we might propose that nucleic information has the pervasiveness and static precision connoted by "genetic," whereas the epinucleic information regulates the manifestation of nucleic potentialities in the dynamic, temporally responsive functioning of actual development.

We can now resolve an earlier debate whether "self-reproducing particle" and "self-sustaining reaction cycle" are meaningfully different concepts (my own argument having been that the act of self-reproduction was just such a cycle). I must agree that there is at least this difference: the tremendous informational complexity of a long linear polymer that is replicated point by point in contrast to the one-bit, yes-or-no alternations we observe in the simpler feedback systems. In fact, the much relied upon criterion of mutability is another aspect of this complexity. A one-bit unit is either present or absent; mutation is the deletion or substitution of one bit in a long string.

At the present time, we know of no other biological system of replication or autocatalytic feedback (short of the whole organism!) that remotely approaches the informational complexity of a polynucleotide, which justifies the contention that the bulk of germinal genetic information is nucleic. On the other hand, we cannot readily visualize a mechanism for the determinate, systematic alteration of nucleotide sequences to account for cellular differentiation. For this reason, chromo-

somal nucleic variation, i.e., gene mutation, has been the least popular element of all genetic models of differentiation. This has left plasmagenes (extranuclear nucleic) and steady states (extranuclear epinucleic) as the chief contenders in the model-building Olympics. But there is now increasing concern for a hypothetical category sometimes overlooked: epinucleic variations of the chromosome. As Ephrussi pointed out, the genetic evidence for epinucleic variation in the nucleus is suppositious (and it cannot actually be quite compelling until we know the nucleotide sequence of a chromosome). However, in paramutation (a phenomenon I hope Dr. Brink will enlarge upon), in the differentiation of amphibian nuclei, and in *Salmonella* phase variation, we have a series of effects that lack the "molar indeterminacy" of ordinary gene mutation, enough to make us suspicious that they might be epinucleic. In the cytochemistry of chromosomes, we have rather more-direct evidence, e.g., in histone versus protamine in different nuclei and in the morphogenetic variation of localized bands of salivary-type nuclei in insects, but it is impossible to assess these qualities as features of cellular *heredity*. Epinucleic chromosome variation is therefore an entirely speculative hypothesis designed to leave some leeway for differentiation in the chromosome without having to invoke balky ideas of determinate changes in nucleotide sequences.

Obviously there is little to say about the details. Epinucleic variation might encompass dynamic equilibria, at chromosome loci, and involving genes and their products (ribonucleic acid protein?) along the same lines as parallel proposals for the cytoplasm. Furthermore, we should perhaps look for variations in nucleic structure that do not alter the fundamental sequence. The compact double helix is, of course, the idealized structure, on whose regular periodicity the X-ray diffraction diagram depends. The diagram cannot tell a great deal about the local deviations from the ideal structure, which may be most pertinent to the present dimension of genetic variation. It can say least of all, by present methods, on the detailed, transient structure of deoxyribonucleic acid (DNA) in the

metabolically active cell. The way in which proteins are coupled to DNA and their point-to-point specificity are open questions and may have much to do with local states. The coupling of polyamines has also been mentioned as another epinucleic variable. What is less clear is how such local states can be replicated along with the informational sequence. However, one can visualize that the accumulation of local specific products at an open region can help to dissociate compactly intertwined helices after replication and make them open as well. This notion supposes that the closed double helix is so compact, i.e., has so little residual chemical activity, that it cannot function without unravelling, either for self-replication or for its heterocatalytic function.

Genetic thinking about development has been dominated by the doctrine of genetic constancy: the conservation of genetic constitution in all the cells of an organism. The fact of differentiation immediately contradicts this generalization, and the paradox has raised a serious intellectual barrier between embryologists and geneticists. I suggest we now try out another *working hypothesis:* the conservation of nucleic information, or at least of chromosomal nucleic information in *normal* development (though we will doubtless have to make some concessions in special cases such as diminution in *Ascaris*).

One doctrine that was brought up in the last session, perhaps too often, was the pleonasm about doing embryology on embryos. My initial thought was to issue a recantation, but after the argument went on a little I began to think that maybe there was something in it after all, in the sense that the limiting factor in the analysis of differentiation is no longer the insufficiency of hypothetical models and partial analogies. Nucleic versus epinucleic and chromosomal versus extrachromosomal are each logically exhaustive classifications of all possible mechanisms, but the assessment of genetic approaches to somatic cell variation depends less on this intellectual exercise than on finding ways to ask critical questions

of embryos, as for example, R. Briggs and T. J. King have done.

Some of the selective methods that have to be devised to cope with populations of somatic cells are foreshadowed by the work of Cotterman and Atwood on the erythron. That there should be some technical difficulties at this stage of analysis is a predictable fact, but we can be certain that diligent concentration on these problems is going to provide some still unsuspected but indispensable tools for the detection and isolation of rare genotypes.

Klein's report and Mitchison's discussion represent the next important steps in the rational construction of a somatic cell genetics. Recombination between pure line cells, whether diploid, or worse if polyploid, will generate heterozygous cells. It will then be an immediate problem of technique to cope with heterozygotes, preferably by inducing segregation to uncover recessive markers. It is now clear that this can be done, though the mechanism still has to be worked out. On the basis of a straightforward tool for detecting rare segregation, we can look forward to development of techniques for inducing and controlling it — by analogy with the effects of ultraviolet light on heterozygotes of yeast, *Aspergillus,* and *Escherichia coli* and on heterokaryons of *Neurospora.*

The most puzzling feature of the results reported here is the asymmetry. Why should a tumor cell of constitution A/S give S/S homozygotes (or S hemizygotes) more often than A/A? Keep in mind that the hybrid was obtained from coisogenic lines that should not differ in much more than the H_2 locus itself. Klein reviewed a number of plausible explanations that further work will doubtless sort out. He left me the opportunity of adding another thought to his list. The very act of selection for a tumor introduces new factors into the cellular genotype. If one of the new mutations should be located on the chromosome carrying the H_2^s allele, only those segregants that continued to carry this chromosome would be detectable as rapidly growing tumors. To test this hypothesis it will be necessary to devise a selective method applicable to

non-neoplastic tissues as well. In the long run, just this sort of analysis will be needed to assay the various genetic bases of neoplasia.

Hauschka's and Ford's analyses of tumor cell populations remind us how much further the cytology of somatic cells has advanced than has their genetics. We can only begin to guess at the full implications of the karyotypic diversity in these populations, and this can only hint at the extent of variation of individual genes.

In the discussions much was said of the esthetic appeal, good manners, laboratory convenience, and karyotypic elegance of the Chinese hamster; regrettably we could not furnish a sample for your inspection at short notice. One assertion has been very puzzling to me, and it is one that could be broached only with a well-differentiated karyotype. This is the viability of *nullisomic* tumor cells. The Chinese hamster has the lowest chromosome number of its taxonomic section, which makes it difficult to invoke polyploidy. There are perhaps three points of view to consider: (1) that some mammalian complements include supernumerary, dispensable chromosomes, like the B chromosomes of maize. This solution might help to clear up the variability of chromosome number, 46, 47, 48, which Stern discussed for man; (2) that the nullisomic cells are the immediate progeny of irregular mitoses and have no capacity for indefinite survival; (3) that the phenogenetic functions of the various chromosomes are well ordered, so that one or more chromosomes simply do not have any genes that are indispensable to cellular viability in the protected environment of an ascites tumor. Such a chromosome is then supernumerary for the tumor cell, though it may be quite necessary for the normal development of the whole animal. The last suggestion has had little explicit support from what we knew of the distribution of gene functions; but it has been revived by the findings of physiologically correlated blocks of genes in bacteria.

Whatever our final conclusion proves to be, a nullisomic karyotype presents special problems of interpretation, apart

from those of interchromosomal balance presented by other types of aneuploidy.

In his account of remarkable studies on mutable genes affecting pericarp color in maize, Brink took pains to disavow their applicability to differentiation, chiefly because of the indeterminacy of the mutations. He was also careful to stress that he was not discussing McClintock's findings on mutable genes, from which she does construct a model. The essential features, as I understand her interpretation, are: (1) that the level of activity of any of a number of loci can be regulated by a transposable "controller"; (2) that these controllers are subject to "changes in local state"; and especially (3) that these changes of state occur not randomly but at sharply circumscribed intervals in developmental time. The last point is the kernel of McClintock's argument: if there are local changes in genes that occur at definite epochs of development, we have a chromosomal event, be it nucleic or epinucleic, that is tied to a developmental clock. The controllers that McClintock uses in her experiments would be pathological deviants (as they must, to be amenable to conventional genetic methods), but they still reflect the morphogenetic cycle of differentiated levels of activity of different genes. Unfortunately, the orderliness with which changes of state occur has not been reported in a detailed quantitative study, and we must rely on a few selected photographs and verbal accounts. Dr. Brink assured us that *his* mutable material did *not* show orderly patterns, and on this basis it is easy to see why these two investigators would draw different conclusions on the relevance of their material to developmental theories. I hope, however, that Dr. Brink will favor us with an account of another system he has been studying, the *R* locus, where he has evidence of orientation of genetic change by one allele acting on another.

The orderliness of normal development that impels us to invoke epinucleic parameters for its cellular genetic analysis stands in vivid contrast to neoplasia. It is therefore reasonably certain that we cannot insist on a unitary theory for the

initiation or progression of tumors. Development of a tumor represents the evolution of a cellular population, in which, in contrast to the fitness of the whole organism, adaptive fitness is reflected in the capacity for ever more rapid and unregulated growth. Thus any incident that initiates or promotes the cell's evolutionary progress to this end must be involved in carcinogenesis: the phenogenetic effect rather than the genetic mechanism tells what role it will play. Present knowledge places no bounds on the scope of mechanisms of variation that might contribute to a neoplastic phenotype, just as we do not attempt to account for the evolution of species by any single mode of genetic displacement. Indeed, it would be surprising if a neoplastic phenotype were always initiated by a single variational event, and we can suppose that the cumulation of several variations (whether by gene mutation, virus infection and transduction, plasmid segregation, recombinaation, or karyotypic upsets) will be necessary before a once normal clone transcends the threshold of malignancy. Once committed to this pathway, a "premalignant" cell may be expected to show accelerated evolution on account of its very augmentation of growth rate and its physiological unbalance. The experimentally designed bacterial populations that Braun displayed may be an introductory primer to the intricacies of the evolution of a tumor.

Before concluding, I would like to take the occasion for an appeal on behalf of the inbred mouse for sophisticated studies of somatic cell genetics. Inbred mice are, of course, quite indispensable for transplantation work, but they have been relatively unpopular in tissue culture. There is no doubt of the anthropocentric glamour of using human tissues, nor can one ignore the investment that has gone into the development of HeLa cells as nearly standard material, and it would be impossible to discount the splendid progress that has been made with it. But when we approach questions of genetic analysis, the unpredictable genetic constitution of HeLa cells, their irreversible heteroploidy and heterozygosity, and the lack of a defined compatible host for retransplantation are likely to

lead to treacherous blind alleys. The type of study that Klein has been doing illustrates the opportunities that await the systematic use of pure line material in tissue culture, which include the facility of constructing known genotypes by conventional mating.

The limitations of human tissues are especially evident when the occasion arises to test cultures *in vivo,* in retransplantation to a fully compatible host. We have now, for example, a confused and contentious picture of the incidence of neoplastic transformation in normal tissues maintained in culture and can never hope to reach a definite conclusion with the use of human material.

OPEN DISCUSSION

AUERBACH [2]: I do not quite see why nullisomics among tumor cells must be a problem. Tumor cells are parasitic cells fed by the host, and even in tissue cultures there may be close cooperation between cells. I know of at least two cases where loss of genetic material could be tolerated by cells in close contact with similar cells. One is in the tapetum of a plant — I forget which — studied by Barber and Callan; the other, the synchronized postmeiotic divisions of the male germ cells of the louse that G. Pontecorvo studied. In the latter case, very large chunks of genetic material could be lost without ill effect on the cell.

STERN [3]: Dr. Lederberg was very much in favor of hybridization of somatic cells, and I would like to give an example where this perhaps has been accomplished. The German botanist, Hans Winkler ('38), made graft hybrids between the two species *Solanum nigrum* and *S. lycopersicum.* In the great majority of cases he obtained chimeras, certain layers of cells being derived from *S. nigrum* and others from *S. lycopersicum.* But after many years of work, he thought he also had two cases to which the term "Burdonen" could be

[2] Charlotte Auerbach, Oak Ridge National Laboratory; on leave from University of Edinburgh.

[3] Curt Stern, University of California, Berkeley.

applied, defined as true vegetative hybrids resulting from nuclear fusion of cells from scion and graft. Only the epidermis of the two plants had Burdo character as indicated by (abnormal!) chromosome counts and phenotype.

I would prefer not to call this sexuality, even though the whole process was there. It seems to me that we perhaps speak of sexuality best when two cells are "made for each other"; in Winkler's cases, it was "just happening."

My other comment is related to Dr. Lederberg's reference to McClintock's finding that there was a specific time in development when the changes in status occurred. She stressed that they were tied to the developmental clock. In *Drosophila,* somatic crossing over is also somewhat tied to time and place of development. Sizes and frequencies of spots vary in various body regions. Even the location of the place of somatic crossing over is correlated with the developmental pattern. Nevertheless, I do not regard this correlation as furnishing a model for genic control of differentiation.

STREHLER [4]: Most of the mechanisms that have been suggested here for genesis of tumors seem to me to have first-order characteristics; that is, you would expect these events (or accidents) to accumulate more or less at a constant rate with respect to time. Yet, the probability that individuals in a population will die of tumors increases exponentially. Is there a hypothesis or an explanation that would logically relate these two phenomena?

KOLLER [5]: I believe it was Mahler who, a long time ago when looking at the increasing incidence of cancer with age in humans, suggested that there must be more than one event in the cell for the transformation of the cell, and he calculated five or six. Following Mahler's suggestion, after a few years, Northerly, from Sweden — who was not a biologist but I believe an engineer who took a fancy to this problem and made an investigation — came to the same conclusion. The problem was then discussed in the British Journal of Cancer by Alde-

[4] B. L. Strehler, National Institutes of Health, Baltimore City Hospitals.
[5] P. C. Koller, Chester Beatty Research Institute.

man and Dorn, and they came to the decision that there must be at least four events. Recently they revised their calculations and stated at least two events.

On the other hand, we have experimental data. Dr. W. E. Heston found that on a linear postulate there might be just one event. But I think it shows how true is Dr. Lederberg's statement that in carcinogenesis we might be dealing with a very complex situation in which everything is involved.

AUERBACH: I find this very unconvincing. It is very well known that mutation rates vary by factors of 10 or perhaps more between cells, depending on their stage, type of metabolism, or age. So I do not see how one can calculate the numbers of mutational events from an age distribution of cancer.

KOLLER: Certainly incorporation is very important. I would like to recommend that you look at the incorporation on which they are based.

KLEIN [6]: I apologize for bringing the discussion back to carcinogenesis. But I have a certain distrust in interpreting curves obtained by pooling data from heterogeneous populations, heterogeneous both with regard to the genetic constitution of the individuals and often also with regard to the tumors studied. Although there is circumstantial evidence from the analysis of such curves that carcinogenesis involves a sequence of several events, conclusive evidence can hardly be obtained from such analyses. On the other hand, there is ample evidence to the same effect; e.g., the studies of J. Furth, W. U. Gardner, H. B. Andervont, and others on endocrine tumors. Biological work of this kind indicates that one is not dealing with simple situations where one can talk about initiation and promotion, about primary and secondary events, or even about a fixed number of events but rather with an evolutionary change involving a sequence of random events, probably different in detail in every single case, and leading gradually toward more and more increased autonomy terminated only by the death of the individual or the experiment.

[6] George Klein, Karolinska Institutet.

It may be argued that endocrine tumors are exceptional. They do not seem to be exceptional to me in any other respect but that, with regard to the systems where they arise, we know something about the growth controlling homeostatic influences whereas we usually know nothing about the other systems.

I should like to add just one more point to the plea Dr. Lederberg made with regard to suitable material for the study of somatic variation and of carcinogenesis, with which I fully agree. I feel that one set of markers that ought to be included in such studies should be of a nature related to the essential phenomenon in malignancy. Hormone dependence of endocrine organs and tumors would be a major candidate at the present stage.

STREHLER: Apropos of Dr. Klein's remark! One would certainly have to agree about the heterogeneity in the human population in which the incidence of tumors is probably best documented. Nevertheless, there is one study by Simms and Berg in which they measured the incidence of tumors quite accurately for a single kind of tumor (in a highly inbred strain of rats) and still found an approximately exponential increase with age. One robin does not make a spring, but I do not think that one can dismiss the extremely elegant fit between log probability of tumor formation and age by simply saying that there is great heterogeneity in the biological material. That may be relevant or it may not be. It may be that there is something about the mechanism of tumor genesis or a change in the organism's resistance, as Dr. Auerbach and others have suggested, that produces this particular exponential kinetics.

STRAUSS [7]: I still wonder whether it is necessary to accept the idea that the polynucleotide base sequence in DNA actually determines gene action. If amino acid sequence determines function, proteins with different sequences should have different functions, and the reverse should also be true. However,

[7] B. S. Strauss, Syracuse University.

there are different amino acid sequences in insulins from a number of species, but these proteins have the same function. Furthermore, separate investigations by Gladner, Schaffer, F. J. Dixon, H. Neurath, D. E. Koshland, and others seem to indicate an identical amino acid sequence at the active site of the enzymes thrombin, chymotrypsin, trypsin, and phosphoglucomutase. All these enzymes have very different specificities. It seems to me that the amino acid sequence in proteins may perfectly well be determined by the sequence of bases in DNA and that this is the source of the species specificity of proteins. But I think that protein activity may be determined by a three-dimensional structure superimposed upon the base order.

Brink [8]: Since Dr. Lederberg made brief reference to it, members of the group might be interested if I said a little more about the curious kind of heritable change that we recently observed at the *R* locus, conditioning aleurone and plant color in maize. The meaning of the evidence for the problem of differentiation is unknown, but the extraordinary lability observed at the locus conceivably is significant in this connection.

When pollen of our standard *RR* strain (self-colored aleurone) is used on *rr* plants (colorless aleurone), the resulting kernels are darkly mottled (*Rrr*). In a strain having the same highly inbred background the mating, *rr* ♀ $\times$ R^{st} R^{st} ♂ (stippled aleurone) yields stippled kernels ($R^{st}rr$). It would be expected, therefore, that when pollen from the heterozygote, RR^{st}, is used on *rr* individuals, one-half the kernels would be darkly mottled and one-half would be stippled. This, however, is not what is observed. The stippled class of kernels is regularly formed, but the other class of kernels expected (dark mottled) does not appear. In place of the latter is a new phenotype ($R'rr$) characterized by weakly pigmented aleurone. Plants grown from the associated $R'r$ embryos transmit the R' allele regularly, although the phenotype of the offspring is shifted somewhat toward that of standard *Rrr* kernels.

[8] R. A. Brink, University of Wisconsin.

Complete reversion of R' to R, however, has not yet been observed.

The change in determinative action of R to the R' level is unique in several respects. (1) It occurs invariably in RR^{st} heterozygotes and not sporadically as is characteristic of mutation; (2) the genetic change is directed rather than random; and (3) the alteration of standard R to R' is partially reversible, likewise with complete regularity. The experiments also show that in heterozygotes with marbled (R^{mb}), an allele distinct from stippled, standard R invariably changes to a third form, which may be designated R'''. Thus the change occurring in R in heterozygotes also is specific and depends on the particular allele in the homologous chromosome.

Tests have been made that exclude the cytoplasm as the basis of the phenomenon. Evidently, intrachromosomal changes at, or near, the R locus are involved. Definitive evidence concerning the stage in plant development at which the inherited alteration in R occurs is not yet available.

Papazian [9]: The interplay of nucleic and epinucleic will be involved when cells that are not "made for each other," in Stern's apt phrase, are crossed. A feature of dual control, nucleic and epinucleic, is that the nucleic is a long-term storage, controlling evolutionary change, whereas the epigenetic is short term.

A necessary requirement for the proper working of this system in evolution is that the epinucleic information be obliterated at each generation and be regenerated *de novo* from the nucleic information of the sperm and egg, cells that are, in this special sense, "made for each other." Only thus can long-term evolutionary change be under absolute and unadulterated control of the organ that is so efficiently designed for just that purpose, the nucleic apparatus.

Progeny from the cross of a liver and a kidney cell will contain a complex of nucleic and still-functional epinucleic combinations nice to unravel.

[9] H. Papazian, New Haven, Connecticut.

(Postscript, August 1958). The genetic basis of antibody formation was mentioned several times during the conference. An antigen may be thought to play either of two roles: *instructive* if the specifications for an antibody are introduced into the cell by an antigen, or *elective* if a preexistent synthesis is potentiated. [The terms "inductive" and "selective" connote a different issue, in population genetics rather than physiology. For example, there are good grounds for inferring an *elective* role of the substrate in enzyme *induction* (Lederberg, '56).]

It would be easier to choose between these roles if we knew more of the molecular basis of antibody specificity. If the γ-globulin molecules of a given animal have the same amino acid sequence, diverse antigens might plausibly instruct their folding in specific patterns (on the controversial assumption that sequence does not already predetermine folding). But how could the miscellany of antigenic substance convey instructions for different specific sequences of amino acids? An elective role for the antigen, on the other hand, would be equally compatible with any hypothesis of antibody structure.

The elective concept has been fully elaborated in Burnet's most recent proposals (Burnet, '57). He writes: "at some stage in embryonic development . . . a 'randomization' of the coding responsible for part of the specifications of gamma-globulin molecules, so that after several cell generations in early mesenchymal cells there are specifications in the genomes for virtually every variant that can exist as a gamma-globulin molecule. This must then be followed by a phase in which the randomly developed specification is stabilized and transferred as such to descendant cells." When a mature lymph cell preadapted to form a given antibody is stimulated by the corresponding antigen, it generates a larger clone of cells actively producing and liberating that antibody. Induced tolerance results from the hypersensitivity of embryonic cells so that prenatal experience of a given antigen abolishes the corresponding clone.

The least congenial feature of this hypothesis is doubtless that a separate clone must be maintained throughout the life

of the animal for each of the potential antibody responses. Whether these are numbered in thousands or billions is debatable, but in either case it is difficult to picture the maintenance of each clone against loss by random drift and especially against the selection for alternative species. To meet this and other objections the following revision was devised in the course of conversations with several participants at the conference, including Burnet. Its main departure is to propose that antibody-forming cells differentiate *throughout life* from a persistent stem line. As a corollary, randomization is a continuing process. The basis for tolerance remains hypersensitivity but of immature *cells* even in the adult animal.

This theme can be elaborated in several different ways. For one, close to Burnet's original, randomization would occur at a definite stage in histogenesis from the stem line. Diverse clones would be recurrently generated but need not survive indefinitely in the absence of the antigen. Hypersensitivity would likewise attend a definite stage of histogenesis. The introduction of an antigen from a time before any cells had matured past this stage would therefore suppress all homologous clones as they arose.

Alternatively, randomization might recur in the stem line itself, subject to stabilization and clonal expansion after a reaction with an antigen. The cell is immature immediately after its transition, hypersensitivity reflecting the reactions of minimal or early antibody.

Nothing has been said of the cytochemical locus of the randomization, nor of a number of other details of its genetic mechanism, such as the total number of possible states (= species of antibodies), their duration and multiplicity in a single cell, and their heritability before, during, and after antigenic stimulation. These and other items will have to be specified for detailed working hypotheses. Nor is tolerance necessarily founded on hypersensitivity, though this is the most plausible interpretation of the role of timing in the response to an antigen.

Current ideas on gene action are founded on the premise that the instructions for protein synthesis are filed in DNA and conveyed through RNA. On this premise, randomization would involve a particular segment of chromosomal DNA or microsomal RNA or both. In randomization, perhaps this patch is assembled at random from available nucleotides, rather than replicated in regular fashion. This mode of synthesis has already been suggested for heterochromatin, and likewise the aberration might be related to dissynchrony in nucleic acid synthesis. Less fanciful interpretations of hypermutability in certain metabolic states of the cell cannot yet be discounted.

The justification of this, as against other hypotheses, awaits experiments on the potentialities of clones derived from single adult cells. For the time being it rests on the proposition, so far uncontradicted, that tolerance can be maintained only in the continuous presence of the antigen, of which prenatally initiated chimerism is the perfect illustration. On Burnet's original version an antigen need suppress the homologous clone only during embryonic life and should be dispensable thereafter. The suggestion that stabilized clones are subject to remutations that must be dealt with to maintain tolerance is tantamount to, and in fact directly provoked, the present revision. Another expectation is that it should be possible to induce tolerance in populations derived from inocula small enough to preclude any cells already reactive to a given antigen. Experimentally, it may be necessary to excite the proliferation of such populations by other antigenic stimuli.

In previous discussion, I stressed the role of epinucleic effects largely for lack of a plausible nexus between embryonic inductions and nucleic information: how could nucleotide sequence be specifically altered by external, non-nucleic agencies? [See Nanney's discussion ('58) of epigenetic regulation, which has now appeared in detailed form.] The system of random hypermutability followed by elective stabilization, as proposed for antibody formation, furnishes another approach to this problem. Nucleic information could be

modified from without if it first underwent a series of random transitions, the apt one being recognized by the reactions of the corresponding products, and other inducers playing the part of antigens. Stabilization of the existing state is an instruction of a kind but far simpler than the predetermination of a nucleotide sequence. In particular, we need not invoke unprecedented reactions of DNA or RNA with external reagents beyond their already imputed functions in protein synthesis.

Antibody formation is the one form of cellular differentiation that inherently requires the utmost plasticity, a problem for which the hypermutability of a patch of DNA may be a specially evolved solution. Other aspects of differentiation may be more explicitly canalized under genotype control. If so, we might revert to the conception of local functional states of various genes whose specificity is unaltered. However, I can no longer insist that these states are epinucleic. Perhaps information that is nucleic but epigenetic should be dignified with another name, if and when it can be proved to exist either in microbiology or morphogenesis.

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